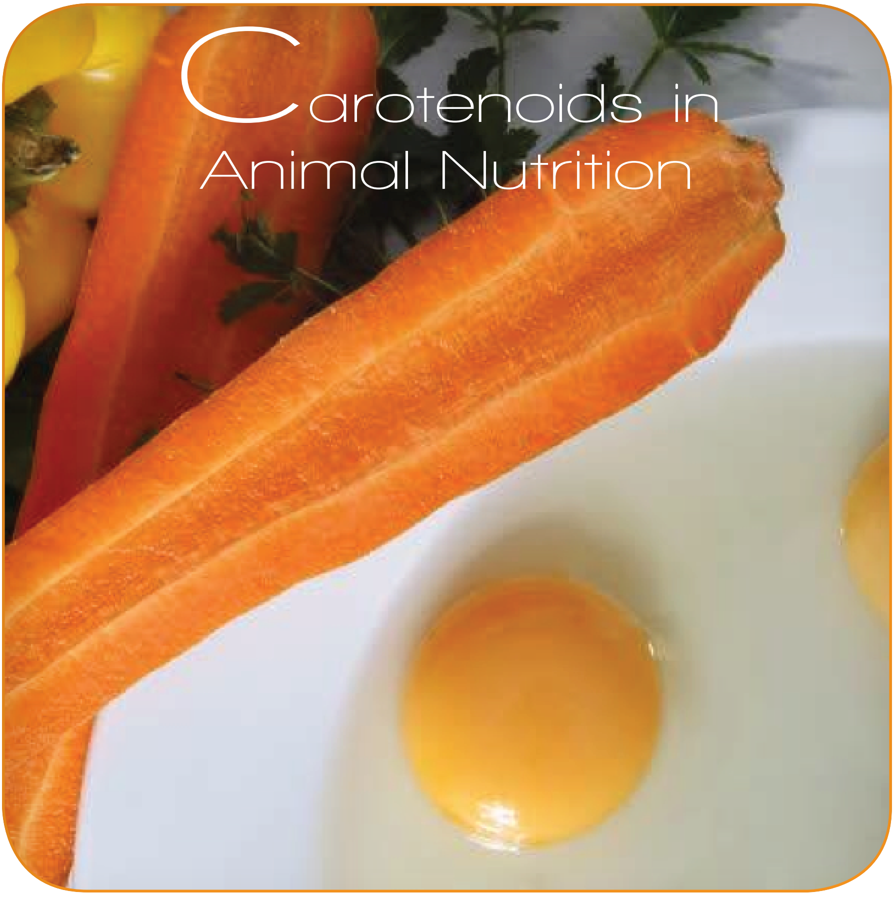
— Fefana Publication —

Carotenoids in Animal Nutrition



fefana

Carotenoids in Animal Nutrition

Contributing companies:

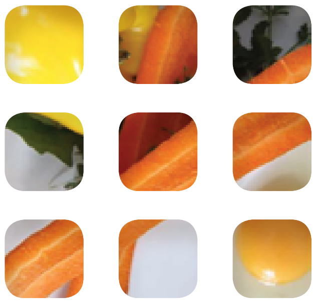
DSM
ITPSA
NOVUS

Authors:

Elkin AMAYA
Philippe BECQUET
Sergi CARNÉ
Silvia PERIS
Pilar MIRALLES

ISBN 978-2-9601289-4-9

Table of Contents



Introduction

Colours are widely present in the animal kingdom, playing an important biological role. For example, in birds, the attraction and mate selection is favoured by bright colours signalling healthy individuals.
These colours come from the animal diet, and are linked to the presence of Carotenoids. Carotenoids constitute the largest group of naturally occurring pigments, showing much diversity in natural distribution, structure and function. They are largely responsible for the variety of yellow to red colours in fruits, vegetables, fungi, feathers of birds, fish flesh, cuticle of crustaceans or insects, as well as aquatic plants and algae.

Carotenoids have always been part of the daily diet of animals and humans. With the development of optimized animal nutrition, the industry created tools to formulate their feed at the best costs to provide animals with required nutrients. Carotenoids are included in the feed as part of the nutrients that support animal health, as well as product quality.

New forms of concentrated carotenoids were developed to help feed formulators providing sufficient carotenoids to the animals at any time, for the benefit of the animals and their end consumers; and they became an integral part of the diet of farmed fish and poultry. With the development of Regulations, those products became feed additives in the EU starting in the 1970’s.

Through this publication, FEFANA aims at presenting an overview of the world of carotenoids with a focus on oxycarotenoids/xanthophylls, their use in animal nutrition and the benefit for the consumers.

Silvia Peris
Chairperson FEFANA Working Group Carotenoids
Didier Jans
FEFANA Secretary General

1. NATURAL OCCURRENCE AND CLASSIFICATION

Carotenoids are naturally occurring pigments (see Figure 1) largely responsible for the variety of yellow to red colours found in plants, algae, fungi, birds, as well as fish flesh, cuticle of crustaceans or insects. For example, the yellowish-orange colour of carrots is due to one of the most familiar carotenoids and vitamin A precursor, β-carotene, from which the name of the whole class of these natural pigments is derived.

All photosynthetic tissues contain carotenoids, although the yellow to red colour from these pigments is frequently masked by the chlorophyll also present in the chloroplasts in higher plants and algae, as well as in the external membrane of photosynthetic bacteria. In fact, carotenoids play a key role in photosynthesis as they absorb light energy and also protect chlorophyll from photo-damage and oxidation caused by light and oxygen. Their major function is thought to be related to their antioxidant properties.

In many animals, during the accumulation of carotenoids from the diet the colours are often masked or modified by the presence of carotenoproteins, which can give different hues of green, red, blue and purple. Carotenoproteins are also frequently found in land plants. As carotenoids are found in abundance in nature, they are also regularly present in human diets. Accordingly, the relevant basal levels of these carotenoids have been identified, with intakes of lutein, β-carotene and lycopene being the highest.

Carotenoids are mostly concentrated in fatty tissues such as internal fat and egg yolk in terrestrial animals, while in fish they are located either in fat or linked to the flesh.

Generally speaking, naturally occurring carotenoids can be obtained from microorganisms, vegetables, and animals, as discussed in more detail below.

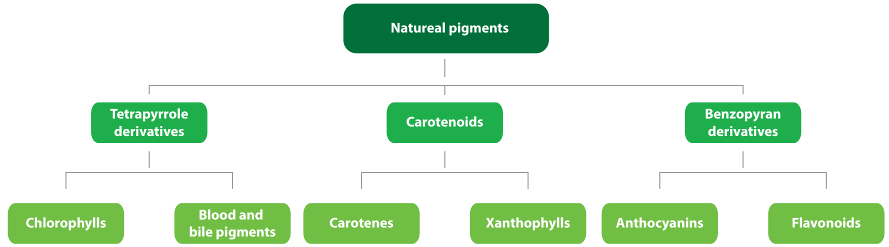


Figure 1 - *Most frequent groups of natural pigments.*

1.1 Natural occurrence of Carotenoids

1.1.1 Microorganisms

The first organisms with the capability to synthesize carotenoids were anoxygenic phototrophic bacteria and oxygenic photosynthetic cyanobacteria. The primary functions of carotenoids were light harvesting and protection against photo-oxidation in the event of gradual increase of environmental oxygen and bright light.

The capability of synthesizing carotenoids was later acquired by plant and algal cells due to their symbiosis with carotenoid-producing bacteria. In the case of terrestrial plants and red and green algae, symbiosis occurred with cyanobacteria.

The diversity of carotenoid structures is particularly large in algae and bacteria, which are able to synthesize a wide range of carotenoids. The vast majority of microorganisms producing carotenoids are photosynthetic.

In regard to carotenoid synthesis, we can differentiate between those produced in small quantities as structural and functional components of the photosynthetic apparatus of the microorganism, and those which are produced in large quantities (carotenogenesis) only when the microorganism is exposed to certain nutritional or environmental stimuli and accumulated in the cell cytoplasm. It is this second mechanism that can make these microorganisms suitable as carotenoid production sources for food and feed industries.

a. Bacteria

A number of bacteria are able to produce carotenoids, which are accumulated in chromatophores and are linked to the cytoplasmic membrane. Within the Archaebacteria, the oldest living organisms on earth, practically only Halobacterium (bacteria tolerant to high salt concentrations) contain carotenoids, in the form of β-carotene, giving a bright red cell suspension. This is the reason why when this organism is present in natural salty lakes they have this characteristic red hue. This is also the case for some thermophilic bacteria, where carotenoids protect them from high temperature damage; they can, for instance, be observed in the characteristic orange rings surrounding hot springs.
On the other hand, Eubacteria, a more evolved bacteria where we find those of interest for animals and human beings, are able to produce a rich variety of carotenoids according to different phylogenetic groups. However, carotenogenesis in these organisms is not efficient enough to become an alternative to other sources. Among bacteria reported to produce carotenoids, only the Gram-negative Paracoccus carotinifaciens is currently used to produce red carotenoids including astaxanthin and canthaxanthin.

b. Algae

Algae are simple unicellular organisms (microalgae) alone or grouped together in colonies, or found forming relatively complex pluricellular tissues (macroalgae). These organisms can be classified into different phyla according to their dominant photosynthetic pigment and include green algae (Chlorophyta), brown algae (Phaeophyta), and red algae (Rhodophyta).
Many different kinds of carotenoids are found in algae. Amongst

them, approximately 30 types have functions connected with photosynthesis, whilst others are intermediates of carotenogenesis or accumulated carotenoids. Despite the fact that numerous microalgae and algae can produce carotenoids, only very few are able to accumulate them in large enough quantities that would be appropriate for mass production purposes.
At present, only the unicellular green microalgae Haematococcus pluvialis is commercially grown at significant levels. This freshwater microalgae is found in temperate regions. Under certain stress conditions such as bright light, nitrogen limitation and high salinity, it is able to form cysts and accumulate massive amounts of astaxanthin in their cytoplasm to such an extent that their colour changes from green to red.
Dunaliella salina is another green microalgae studied for their potential commercial cultivation. It is adapted to extremely saline conditions where few organisms can survive. To protect from the intense osmotic and light challenges in such environments, these organisms produce large quantities of β-carotene. This species shares a similar salt tolerance with the bacteria Halobacterium, although the typical red hue in salt lakes is mostly due to the latter.

c. Fungi

Compared to other groups of organisms, several fungal groups have the ability to produce and accumulate remarkably high levels of carotenoids intracellularly, a property that they share with certain microalgae. Different species from the phyla Chytridiomycota, Zygomycota, Chytridiales and Basidiomycotina have been described as showing carotenogenic properties. Among these species we can highlight Cantharellus sp, Phaffia rhodozyma, Blakeslea trispora and Rhodotorula rubra. The mushroom Cantharellus cinnabarinus (Figure 2) is one of the best example of occurrence of orange-red oxycarotenoid canthaxanthin in nature.

Figure 2 - *Cantharellus cinnabarinus, example of a food source containing canthaxanthin.*

Phaffia rhodozyma (asexual stage of Xanthophyllomyces dendrorhous) is a red yeast from the class Basidiomycetes, whose most remarkable feature is the pink to orange colour of its colonies due to the synthesis of the carotenoids astaxanthin and

β-carotene, coupled with its ability to ferment different sugars. This feature is singular among yeast species and has received considerable commercial interest as a biological source of astaxanthin. In nature, P. rhodozyma is mainly found in sugar-rich slime fluxes of certain broad-leafed trees. Rhodotorula is another genus of red yeasts which has been described as a natural source of β-carotene, although its relevance is very limited compared to other microorganisms.
Blakeslea trispora is a fungus from the order Phycomycetes. The speciality of these fungi is that they are able to increase the amount of carotenoids synthesized when the two sexual mating types are cultured together. The main carotenoids obtained are lycopene and β-carotene.

1.1.2 Fruits and Vegetables

Quantitatively, the majority of naturally occurring carotenoids are present in fruits and vegetables. In terrestrial plants, carotenoids are found in highest quantities in the leaves, other green parts, flowers, roots and seeds. Although the content and types of carotenoids found within green tissues are relatively uniform across plant species, there is much more variability in non-green tissues, such as flowers, fruits and seeds.

Carotenoids are accumulated in chloroplasts and chromoplasts. As a result, carotenoids give a wide range of yellow-orange-red colours to fruits and petals of flowers whereas in the green parts they remain masked due to the presence of chlorophyll. In the case of fruits, the chloroplasts in green unripe fruits gradually change into chromoplasts on ripening. Typical examples are the tomato and red pepper. The presence of carotenoids is fully expressed in many flowers as they are found accumulated in chromoplasts in the absence of chlorophyll.

Carotenoids found in terrestrial plants mainly correspond to red and yellow xanthophylls such as lutein, zeaxanthin, capsanthin, violaxanthin or neoxanthin, all of which belong to the xanthophyll cycle and are involved in protection of photosynthesis. Xanthophylls can be found in feed substances that contain green leaves such as alfalfa as well as cereal seeds, particularly maize (Figure 3).

Figure 3 - *Yellow maize.*

In addition, the Marigold flower (Tagetes erecta) (Figure 4) and the red pepper/paprika (Capsicum annuum) (Figure 5) are good examples of the natural occurrence of lutein yellow and capsanthin red xanthophylls, respectively. A wide variety of vegetables are rich in carotenes e.g. lycopene in tomatoes and β-carotene in carrots and sweet potatoes.

Figure 4 - *Marigold flower, a source of lutein for the food and feed industry.*

Figure 5 - *Capsicum annuum (sweet paprika) a source of capsanthin for the food and feed industry.*

1.1.3 Animals

With the exception of some species of aphids, animals and humans do not synthesize carotenoids de novo and must obtain them from microorganisms, plants, and other organisms in their diet. In the case of animals, ingested carotenoids may be deposited in tissues either without modification or after being subject to metabolic changes. When referring to carotenes, those with provitamin features can be converted to vitamin A.

Carotenoids as such are mainly responsible for the yellow to red colours in birds, fish, reptiles and insects. They can also form complexes with proteins and lipoproteins producing an even wider range of colours, including green and blue hues. In addition to their pigmentation abilities, carotenoids have also been shown to have excellent antioxidant properties, often helping to avoid oxidative stress, which poses health risks, especially for immunity and reproduction.

In marine animals, carotenoids can be found in marine sponges, sea urchins, shells of krill and prawn, as well as in the scales of a variety of fish. The characteristic pink to orange pigmentation of salmon and trout flesh is also derived from carotenoid deposition, mainly astaxanthin, which is obtained from krill in the diet.

The most obvious case in wild animals is the diversity of carotenoids found in bird feathers and skin, as well as in reptile and amphibian skin. These animals owe their colour to carotenoids in their diet. Table 1 presents some of the main sources of carotenoids in nature.

Table 1 - *Sources of some carotenoids in nature.*

Representative carotenoids	Sources of carotenoids and their metabolites
Astaxanthin	Adonis annua flowers, fungi, algae, bacteria, shrimp (e.g. via transformation of β-carotene present in their diet)
β-apo-8'-carotenal, β-apo-8'-carotenoic acid	Grass, alfalfa, green plants, citrus fruit
β-carotene	Carrots, fungi, algae, bacteria
Canthaxanthin	Crustaceans, fungi (Cantharellus sp.), algae
Capsanthin, Capsorubin	Red pepper (Paprika - Capsicum annuum)
Citranaxanthin	Citrus fruit
Cryptoxanthin	Fruits, pumpkin, yellow maize, seaweed, peaches, nuts
Lutein, Zeaxanthin	Yellow maize, Marigold flower, alfalfa
Lycopene	Alfalfa, tomato, watermelon, pink grapefruit, papaya, guava, rose hip, fungi

1.2 Chemical structure and classification

Carotenoids were named by their discoverers for some special property or for their source, e.g., carotene (from carrots), cryptoxanthin (hidden pigment), and zeaxanthin (from maize, Zea mays). The major carotenoid subgroups are carotenes and xanthophylls (oxycarotenoids).

Carotenes contain no oxygen in their molecular structure, which means they are pure hydrocarbons, usually orange in colour. The best known representative of this group is β-carotene. On the other hand, the yellow to red carotenoids, known collectively as xanthophylls, are characterized by their oxygen functions (oxycarotenoids).

The main chain of the molecule from naturally occurring carotenoids consists of eight 5-carbon terpenoid joined units, so that the sequence is reversed at the centre. This results in a parent C40H56 hydrocarbon skeleton or backbone conjugated by alternating double and single bonds. The double-bonded structure constitutes the striking and characteristic pigmenting trait. Further modifications at the molecule ends occur during biosynthesis.

Isomerism can occur in the event of rotation of C=C double bonds throughout the molecule, obtaining the so-called trans and cis isomers. Isomers differ not only in their melting points, solubility and stability, but also in ultraviolet characteristics. Carotenoids in nature are predominantly composed of the linear, extended conformation of trans-isomers, which are more thermo-stable than cis isomers.
The size and shape of the carotenoid molecules have key implications on their colouring properties. All coloured carotenoids show all-trans configuration. The molecular structure of the carotenoids and the presence of the extended conjugated double bonds cause the presence of highly delocalized electrons. As a result, the double bond central structure in carotenoid molecules acts as the light-absorbing chromophore and is responsible for the intense yellow to red colours observed.

The number of conjugated double bonds varies from 7 to 15. In fact, at least 7 conjugated double bonds are needed for the carotenoids to impart colour, and the colour deepens as the number of conjugated double bonds increases. The conjugated double bond carbon chain structure of carotenoids is also responsible for the ability of carotenoids to quench damaging free radicals that cause tissue oxidation. Thus, carotenoids have also been acknowledged to have potent antioxidant properties. As a group, carotenoids are extremely hydrophobic with little solubility in water, although cis-isomers are more readily solubilised.

Carotenoids can be found in their free form or esterified with a variety of organic fatty acids such as palmitic, linoleic, estearic, etc. Ester forms are more stable as they are less subject to oxidation. However, they are also less bioavailable as they are not efficiently absorbed through the intestinal epithelia unless they have been previously converted into their free form in the intestines.
Carotenoids can be divided into two main groups: carotenes, and oxycarotenoids or xanthophylls. Carotenes are exclusively made up of hydrocarbons whereas xanthophylls are carotene derivatives containing several oxygen atoms.

1.2.1 Carotenes

Their molecular structure is exclusively made up of carbon and hydrogen forming a polyunsaturated chain with the chemical formula $C_{40}H_{56}$. There is a variety of carotenes occurring in nature, although those relevant to animal nutrition are mainly β-carotene and lycopene. β-carotene exhibits the reference pro-vitamin A activity among carotenoids

1.2.2 Xanthophylls

Also known as oxycarotenoids, this group is essentially derived from the carotene hydrocarbon parent structure where 1 to 4 oxygen-containing functional groups such as hydroxyls (-OH), ketones (=O), or carboxyls (-COOH), are added. These functional groups are added to one or both ends of the molecule. The general chemical formula of xanthophyll is $C_{40}H_{56}O_{1-4}$. The presence of the oxygenated groups makes these carotenoids more water soluble than carotenes. Some of the carotenoids in this group are lutein, astaxanthin, canthaxanthin, capsanthin, citranaxanthin and ethyl ester of β-apo-8'-carotenoic acid.

1.2.3 Biosynthesis pathway in nature

As already mentioned, carotenoids are synthesized in nature by photosynthetic plants, algae, bacteria, and some fungi (see Figure 6). Animals can metabolize carotenoids in a characteristic manner, but they are not able to synthesize them. The total global biosynthesis of carotenoids in nature is estimated to be in excess of 100 million tons per year.
Although differences between higher plants and microorganisms can be found, all carotenoids are biosynthesised from basic C5 terpenoid units (isopentenyl diphosphate [IPP] and dimethylallyl diphosphate [DMAPP]), leading to the parent C40 hydrocarbon skeleton from which all the carotenoids are derived. This skeleton

undergoes a number of modifications including cyclization, hydrogenation, dehydrogenation, introduction of oxygen-containing functional groups, rearrangement, double-bond migration, isomerisation, chain shortening or extension, etc, which may occur in one or both ends of the molecule and leading to the great diversity of naturally occurring carotenoids.
As previously described, oxygenated xanthophylls originate from non-oxygenated precursors of carotenes. In fact, lycopene can be considered as a major precursor in nature of the rest of existing carotenoids. Each biosynthesis step within the pathway is catalyzed by a particular enzyme. The presence of these enzymes is dependent on the expression of different genes. Therefore, the profile of carotenoids occurring in organisms also depends on their genetic profile.

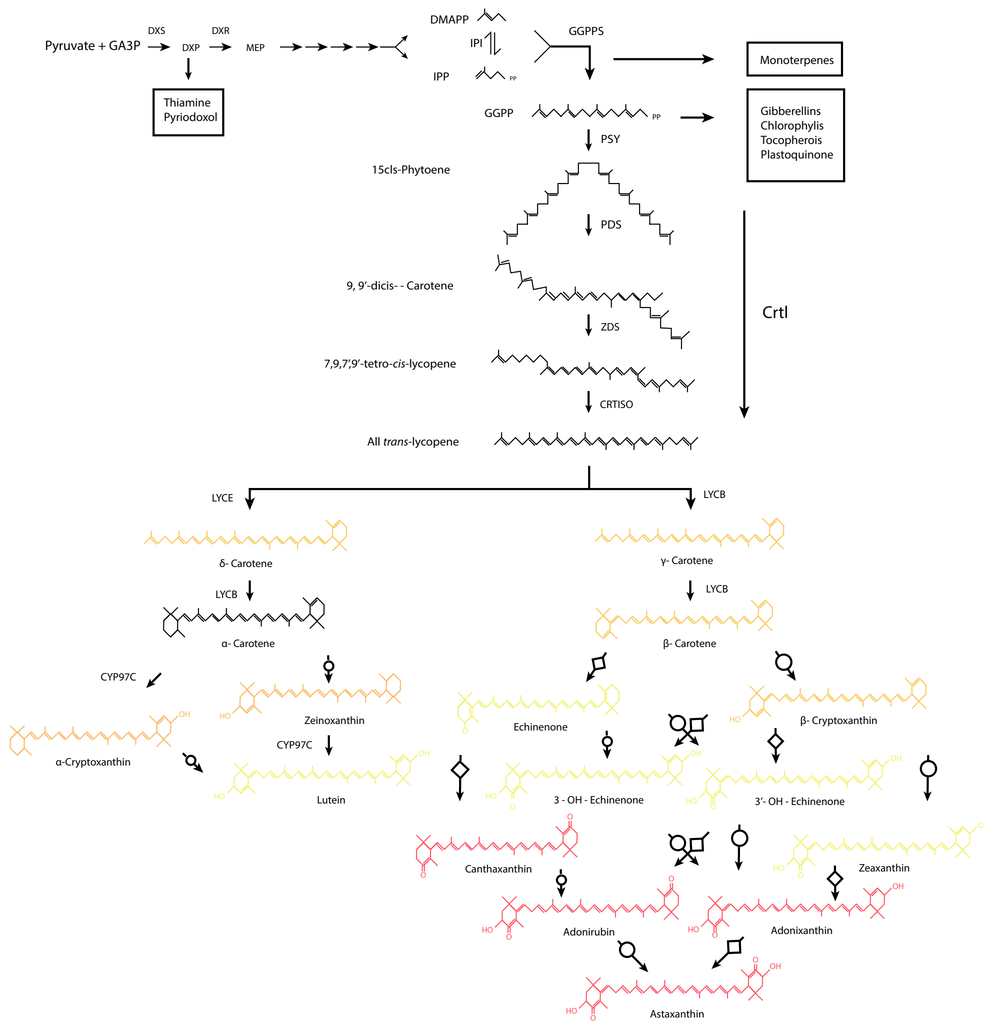


Figure 6 - *Carotenoid biosynthesis pathways in plants. [Farré et al. 2010 Plant Science 179: 28-48]*

2. ROLE OF CAROTENOIDS

Nature relies on a variety of natural compounds (e.g. Carotenoids, chlorophylls, etc.), for the colouration of living animal and plant matter. Carotenoids are widely found in nature, both in animals and plants (refer to Chapter II) and have different functions. Their properties make carotenoids one of the most important biological elements in nature. They are synthesized by all photosynthetic organisms, some aphids, some bacteria and fungi alike. More than 750 carotenoids have been identified and approximately 60 of them are precursors of vitamin A, β-carotene being the most important.
In plants, carotenoids function as antioxidants, hormone precursors, colourants and essential components of the photosynthetic process. One characteristic of carotenoids is their ability to absorb light in regions of the visible spectrum where chlorophyll is not a very efficient absorber. They absorb light and transfer some of the light energy they absorb to chlorophyll, which then uses this energy to drive photosynthesis. Secondly, they can protect plants which are over-exposed to sunlight. They protect against irreversible photodestruction of the photosynthetic apparatus. Carotenoids are essential for the survival of photosynthetic organisms, as they are lipophilic antioxidants which protect cell membranes against oxidative damage. They do this by harmlessly dissipating excess light energy which they absorb as heat. In the absence of carotenoids, this excess light energy has the potential to destroy proteins, membranes, and other molecules.
Animals are generally unable to synthesise carotenoids and require a dietary intake of plant products to meet daily demands. Carotenoid derivatives promote health, improve sexual behaviour (colour signalling) and are essential for reproduction. Some dietary carotenoids can be cleaved to provide vitamin A and are valuable for many physiological functions (e.g. antioxidant activity, immunostimulants, yolk nourishment to embryos, photoprotection).

2.1 Colour

The most obvious function of carotenoids in animals is to give colour. The hue and relative colouring efficiency of carotenoids is dependent on their individual chemical structures. The wavelengths of the colours of the carotenoids used for egg yolk and broiler pigmentation fall between 400nm and 600nm within the visible range of the colour spectrum. To the human eye, such compounds are yellow to red in colour (Figure 7).

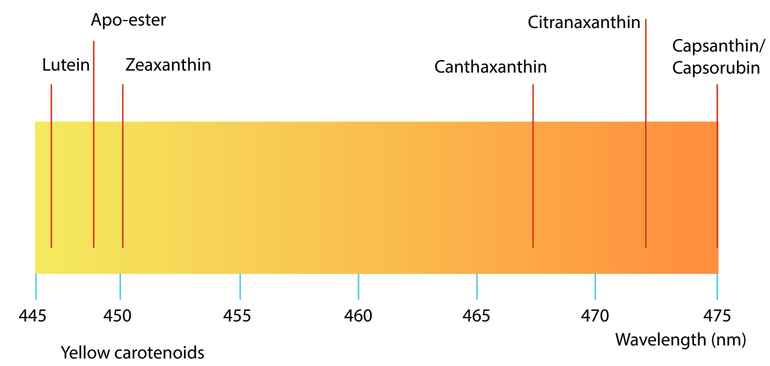


Figure 7 - *Wavelengths of various carotenoids.*

a. Birds

In birds with ornamental coloured plumages, the development of carotenoid pigmented plumage (yellow, orange and red) and some of the brighter hues are due to their diet, since they eat vegetables rich in carotenoids.
The colours in the feathers are formed in two different ways, either from pigments or from light refraction caused by the structure of the feather. In some cases feather colours are the result of a combination of pigment and structural colours. The greens

of some parrots are the result of yellow pigments overlaying the blue-reflecting characteristic of the feathers. In general, red, orange, and yellow to violet colours are the consequence of feathers colourisation via carotenoids. Some examples include the flamingo and the Scarlet Ibis (Figure 8). A flamingo's pink or reddish feather, leg, and facial colouration come from a diet high in alpha and beta carotenoid pigments, including β-carotene and canthaxanthin. The richest sources of carotenoids are found in the algae and tiny animals such as shrimp, molluscs, and insect larvae that live in the mud at the bottom of shallow pools that make up the bulk of a flamingo's diet. For that reason, zookeepers rely on supplementing diets with carotenoids, such as canthaxanthin or similar carotenoids to keep flamingos or Scarlet birds as colourful as in the wild.

Figure 8 - *Flamingo and Scarlet Ibis.*

Another example of feather pigmentation due to carotenoids are different songbirds such as the yellow warbler or the common yellowthroat, which owe their yellow plumage coloration to the lutein in their diets (Figure 9).

Figure 9 - *Parus caeruleus (Eurasian blue tit) and Parus major (great tit). [Courtesy pictures of: A. Borràs and J. C. Senar (Natural History Museum of Barcelona)].*

Birds are also able to accumulate carotenoids from the diet in the egg yolk very efficiently. Lutein, zeaxanthin, β-carotene and canthaxan-

thin are important carotenoids deposited in their eggs, which results in a wide range of yellow to orange hues in egg yolk.

One important factor affecting pigmentation (feathers, beak, legs, fat, egg yolk, etc.) in bird is animal health. Illness greatly reduces the absorption of carotenoids. Normally, an unhealthy or diseased bird would be pale since it is less efficient in absorbing and depositing carotenoids throughout its body. In addition, unhealthy animals would metabolize the already accumulated carotenoids to activate their immune defences.

b. Fish and Crustaceans

Most people consider salmonids as a "red" fish. The flesh from wild salmon from oceans and rivers is often red, pink or orange, in varying degrees. The major carotenoid in wild salmonids is astaxanthin which originates from the feed they live on, small crustaceans or other fish with small crustaceans in their digestive system.

Many marine and freshwater animals, including fish and crustaceans, owe their bright colouration to carotenoids as well (Figure 10). Just like poultry and mammals, aquatic animals cannot bio-synthesize carotenoids de novo, they depend entirely on feed for their supply of carotenoids. The carotenoids in crustaceans are mainly from algae; and in fish from plankton, from other fish with small crustaceans in their digestive system or from small crustaceans they eat.

Figure 10 – *Shrimp.*

Carotenoids in crustaceans are frequently linked to protein molecules, and may provide blue or green colour. Dietary carotenoids, among them astaxanthin, are the responsible for the characteristic red colour of shrimps and other crustaceans when cooked. This red colour originates from the release of astaxanthin from the carotenoproteins when denatured by the heat of cooking.

2.2 Reproduction

Carotenoids fulfil many processes that are essential for normal growth and development in plants, but they are also responsible for the breath-taking variety of yellow to red colours we see in leaves, fruits, vegetables and flowers as well as to provide several aromas in plants. Moreover, such visual diversity helps to attract pollinators and encourages herbivores to distribute seeds aiding plant reproduction.
In nature, the majority of flowering plant species only produce seeds if animal pollinators move pollen from the anthers to the stigmas of

their flowers. Without this activity, many species and processes within an ecosystem would collapse. Pollination is crucial for the maintenance of biodiversity. Approximately 80% of all flowering plant species are specialized for pollination by animals, mostly insects. Plants use chemical components to attract pollinators to visit flowers. This interrelationship between the two is governed by biochemical factors such as colour, scent and qualities of pollen and nectar.
In addition, carotenoids are also the precursors of a range of aromas. As a pollinating animal approaches a flowering plant, one of the signals it receives is an olfactory one, from the flower scent. These volatile scents play an important role in attracting pollinators and herbivores to the plant.
Furthermore, as one might expect from the amazing diversity of colours and patterns exhibited by more than 9,000 bird species found in the world, birds can see colour. In fact, they can discriminate a greater variety of colours than humans; as some birds can see into the ultraviolet range, visual stimuli are important. This has to do with the fact that here other stimuli are less well developed. Contrary to mammals, smell and taste stimuli play a minor role.
Thus, carotenoids are key-elements for breeding success of birds. In fact, linked to their colour, birds communicate their reproductive fitness to prospective mates by providing a vibrant and bright plumage, a sign of being successful at obtaining both a sufficient quality and quantity of food. Carotenoids are fat soluble compounds, which means that theirabsorption is affected by the level of fat in the diet. As a consequence, a diet low in fats may cause feathers to be improperly coloured since the animal is not absorbing enough carotenoids. The more colour and more brightly coloured a male is, the greater the likelihood of attracting a mate. The colourful plumage and shiny red comb of cocks can also act as a deterrent to possible rivals when trying to win the favour of hens and, at the same time, should attract the attention of the other sex.
Also in crustaceans, the cosmetic nature of accumulating carotenoids onto their shells creates a visually more attractive breeding partner appeared to enhance the frequency of their courting behaviour during mating seasons.

An additional function related to reproduction is the pro-vitamin A activity of some carotenoids, of central importance without any doubt. Animals are not capable of synthesizing vitamin A, which is essential e.g. for vision, growth, reproduction, resistance to various bacterial and fungal diseases, normal development of the skin and mucosa.
Besides provitamin A and colour signalling function in animals, carotenoids are also involved in a number of further physiological functions. Of particular interest in this connection is the beneficial effect of carotenoids on the endocrine system in respect of gonadal development and maturation, of fertilization, of hatching, viability and growth, particularly in fish and crustaceans and on the reproductive process in a variety of further animal classes and species, e.g. birds, cattle, horses and pigs. As an example, egg yolk carotenoid levels in wild birds may be five times as high as they are in farmed poultry:

- 10-15 µg/g in farmed poultry - 60-70 µg/g in seagulls - 100 µg/g in cormorants - 150 µg/g in pelicans.

The relatively high carotenoid levels in egg yolks may be beneficial to the developing embryo, especially during the first steps of incubation. This can be accounted for by the antioxidant properties of certain carotenoids. The carotenoids stored in the egg yolk play a crucial role in the development of chicken embryos. The very high metabolic rate of the embryo produces a relatively high amount of free radicals, which may have negative effects. The carotenoid antioxidant effect prevents these negative effects. Such protection allows optimal development of the embryo and ensures a good survival rate during the critical phase that follows hatching.
Moreover, carotenoids are thought to play specific roles in avian embryonic development. For example, research has shown that canthaxanthin supplementation of breeder diets significantly increased the anti-oxidative status in the egg yolk as well as the hatching rate of chicken eggs. It has been found that the concentration of lutein and zeaxanthin in hens and embryonic tissue are associated with increased resistance to oxidative stress and decreased lipid oxidation. Lutein in hen's diet has also been shown to affect the immunocompetence of chicks. In songbirds, dietary lutein and zeaxanthin in the mothers have been demonstrated to produce significant improvement on eggs hatchability and progeny survivability.

2.3 Others

a. Immunity

Carotenoids have demonstrated to affect innate, humoral and cell-mediated immune responses in a variety of models, including birds, dogs, cats, fishes and humans. The fact that carotenoids are localized in immune tissues and cells is the first evidence that suggests that they have a role on immunomodulation.
In this sense, it has been shown that birds fed dietary carotenoids, including lutein, canthaxanthin and β-carotene, have higher levels of carotenoids in the bursa and the thymus than do birds fed no dietary carotenoids. Also monocytes, a type of immune cell, incorporate lutein, and their content is influenced by dietary lutein level.
Thus, carotenoids are present and deposited into immune organs or cells, showing different functions. According to this, it has been demonstrated that, for example, lutein has a direct effect on immune system, as seen through the increases in T cells and macrophages in response to a swelling process in birds, dogs and cats, as well as increased amounts of antibody titers against an antigen in hens and dogs. Linked to its immunomodulation effect, lutein has also been widely reported to act as an anti-inflammatory natural substance in poultry. Carotenoids are also important on improving immunity in marine species. It has been shown that salmonids having a higher content of astaxanthin are more resistant to bacterial and fungi diseases. In relation to crustaceans, astaxanthin has shown to increase the survival rate. A study in shrimps demonstrated that there is a significant decrease in mortality of adult shrimp fed a carotenoid-enriched diet in comparison with individuals receiving carotenoid-free diets.
The effects of various regimes of carotenoid-supplemented diets in prawns (P. japonicas) showed that survival was higher in prawns fed with a diet supplemented with astaxanthin. A positive correlation be-

tween survival and pigment concentration of tissues was even established. It was suggested that carotenoids function as an intracellular oxygen reserve, which allow crustaceans to survive under hypoxic conditions common in pond cultures.

b. Health implications in humans.

A vast number of studies have investigated the role of a wide range of dietary carotenoids in the prevention of several chronic diseases. Thus, carotenoids, whether provitamins A or not, have been credited with other beneficial effects on human health: enhancement of the immune response and reduction of the risk of degenerative diseases such as cancer, cardiovascular diseases, cataract, and age-related macular degeneration (AMD).

The action of carotenoids against diseases has been attributed to an antioxidant property, specifically to their ability to quench singlet oxygen and interact with free radicals. This ability has been linked to the conjugated double bond system which stops oxidation chain reaction. However, other mechanisms have been reported: modulation of carcinogen metabolism, inhibition of cell proliferation, enhancement of cell differentiation, stimulation of cell-to-cell communication, and filtering of blue light.

The macula lutea of the human eye is the center of the human retina responsible for our ability to perform high visual acuity tasks such as reading, driving, and recognizing faces, and where very high concentrations of lutein and zeaxanthin are found. In fact, these two xanthophylls and their metabolites collectively constitute the macular pigment. The retina and lens suffer oxidative damage, and antioxidants are implicated as protective. Lutein and zeaxanthin have been implicated as being protective against AMD and cataracts.
AMD is the leading cause of irreversible visual loss in the elderly in the developed world. In the past decade, treatment of advanced AMD has improved, but it is still of paramount importance to try to prevent or at least delay its onset. Increasing epidemiological evidence indi-

cates that individuals with high dietary intakes of lutein and zeaxanthin have significantly lower risk of visual loss from AMD.

For more than two decades epidemiological studies have associated the high consumption of carotenoid-rich fruits and vegetables to a lower risk of human cancers. These studies have demonstrated an inverse relationship between the levels of carotenoids in blood plasma and the incidence of some kinds of cancer. It has also been observed that various carotenoids inhibit the proliferation of different types of carcinogen cells in vitro. The in vivo anti-tumor activity of several carotenoids against colon carcinogenesis in a rodent model has been reported. Therefore, based on all of the experimental data available to date, it appears that dietary carotenoids can collectively serve as excellent chemoprotective agents in disease prevention.

2.4 Mimicking nature

Taking advantage of their colouring properties, commercial poultry are routinely fed with carotenoids to enhance the pigmentation and the marketability of their eggs and meat. Quite rightly, most consumers associate colour with quality and health. Indeed, the appearance and colour of a product influences consumer product choice in several ways; uniformly well pigmented products are generally preferred by consumers, as indicated by numerous studies. These two parameters are associated to quality of the product.

a. Poultry industry

The origins of pigmentation of egg yolk and poultry are diverse, but they are mainly related to health aspects. They are grounded in the traditional familiar farming systems. These systems involve a semi-extensive rearing system where animals have access to grass, maize and high carotenoid diets. Carotenoids from these diets are responsible for the pigmentation of egg yolk, skin (and fat) as well as legs, beak, comb and feathers. When birds fail to consume these xanthophylls, the hues of their egg yolk or skin become paler. Some poultry diseases negatively affect pigmentation of yolk and skin, such as coccidiosis, CRD (Chronic Respiratory Disease), enteritis or Newcastle disease. Therefore, a uniform and good pigmentation generally means good health and good practical hygienic conditions.

For marketing of poultry products, in many countries of the world, a bright yellow or yellow-orange colour in egg yolk (Figure 11) and broiler skin (Figure 12) is often associated to freshness, health and has become an indicator of high quality products.

Figure 11 - *Egg yolk pigmentation.*

Animals cannot synthesize carotenoids and the colours demanded by consumer cannot be achieved only with the sources of xanthophylls in form of raw materials in feeds. Therefore, carotenoids began to be added to feed to achieve the desired colour and conform to customer preference.

The colour finally perceived by the consumer is mainly a function of both the quantity and the type of the carotenoids in the diet.

There are many factors that influence the colouration of poultry tissues and can be classified as: (1) external, those that affect the carotenoid before the animal consumes it; or (2) internal, the ability of the animal to metabolize (genetic ability) and deposit subcutaneous fat. Also, health is determinant.

For a desirable broiler skin and egg yolk colour, a mixture of yellow and red xanthophylls is recommended. Main carotenoids are lutein, zeaxanthin, and apo-ester (the ethyl ester of β-apo-8’-carotenoic acid) as yellow carotenoids and canthaxanthin and capsanthin as red carotenoids. They all can be blended to produce a wide range of yolk colours at lower total xanthophylls levels than feeding yellow supplements alone. However, if red carotenoids were used solely, a pinkish yolk would result. Therefore, it is important to get the right combination, which should also take into account the native xanthophylls present in the feed ingredients of the diet.

Figure 12 - *Broiler pigmentation.*

b. Aquaculture industry: fish and crustaceans

For marketing of aquaculture products, in many countries of the world, carotenoids are added to feed. Salmonids and crustaceans are major contributors to the world production of farmed finfish and shellfish. To meet consumers' demand for pigmented flesh and exoskeleton of fish or crustaceans, intensive aquaculture diets are fortified with carotenoids. The colouration of salmonids flesh (Figure 13) or shrimp carapaces is considered a criterion of quality.

Figure 13 - *Salmon flesh pigmentation.*

It has been found that in marketing for farmed salmon a pink-red colouration of the flesh which fits the consumer's image of wild salmon flesh is necessary. To achieve this, carotenoids are added to fish feed, so that farmed salmon ingest it in the same way as wild salmon. In these cases, astaxanthin and its salts are generally used alone or more rarely in combination with canthaxanthin to enhance the pink-red colour of the flesh.
The efficacy of pigmentation depends on factors such as: form

and concentration of carotenoid source, diet composition and dietary fat content and quality, fish size and physiological state, state of sexual maturation and genetic background.
A similar approach is happening with farmed crustaceans, for which a pink-red colour of the exoskeleton and/or external part of the flesh (when boiled), is desired. This colouration can be achieved by taking into account the inherent carotenoids of the ingredients of the diet and the supplemented ones, usually astaxanthin, β-carotene and/or canthaxanthin.

c. Pets: ornamental birds and fish

Carotenoids are added to feedingstuffs of ornamental birds, and fish with a view to colour them. For ornamental fish, main carotenoids used are astaxanthin and its salts and canthaxanthin.
In addition to the use of carotenoids in zoo animals, β-carotene, canthaxanthin and lutein-zeaxanthin are frequently used in feedingstuffs for canaries and other songbirds, giving a wide range of hues to feathers from bright yellow to orange-red. β-carotene and canthaxanthin are particularly used in colour feeding Red Factor Colour Bred Canaries to maintain their rich red plumage. The proper ratio is to give the birds half canthaxanthin and half β-carotene. This way the birds will develop bright red feathers.

3. PROCESSING OF CAROTENOIDS AS FEED ADDITIVES

The quality of food is associated with many aspects (colour, flavour, texture, and odour), although colour is considered in humans as the most relevant due to its appealing nature. In fact, undesirable colours are frequently related to undesirable flavours or even potential danger.

Many farm animals and animal products contain carotenoids. As animals do not synthesize carotenoids, they need to be present in sufficient levels as components in feeds. Feeds need to provide a balanced diet, specific for each animal type in order to achieve a high performance. Historically, animal production involved mainly fresh feed, which provided the basic macronutrients but lacked sufficient quantities of carotenoids, vitamins and minerals. Carotenoid depletion becomes more serious after feeds such as hay, straw and fodder are stored or processed. During winter and towards spring, when fresh feeds were not available, incidences of carotenoid and vitamin deficiency were common. This was often manifested by low yields, reduced fertility, sickly young, increased proneness to disease and higher mortalities.

With the demand of better animal nutrition, carotenoid supplementation in the animal diet can overcome these problems caused by deficiency. Today, carotenoids are commercially produced for feed, and can either be obtained by total synthesis, extracted from natural sources or biosynthesised by microorganisms (Table 2).

Table 2 - *Technical processes of carotenoids in the feed industry.*

Carotenoids	Total synthesized	Extracted	Biosynthesized
Astaxanthin	×		×
Astaxanthin dimethyldisuccinate1	×		
β-apo-8’-carotenal	×		
β-apo-8’-carotenoic acid ethyl ester (apoester)1	×		
Bixin		×	
Canthaxanthin	×		×
Capsanthin		×	
Citranaxanthin	×		
Lutein		×	
β-carotene	×		×
Zeaxanthin2	×	×	

[1]esterified form of the naturally occurring carotenoid, 2purified forms can also be obtained by full isomerization from the naturally occurring lutein

3.1 Nature identical carotenoids prepared by total synthesis

Naturally occurring carotenoids are produced by total synthesis when they are present in nature but not efficiently obtained from natural sources and/or where a relatively short synthesis pathway is required (e.g. canthaxanthin, astaxanthin, β-carotene) which makes them technologically feasible and cost-effective. Typical chemicals schemes of carotenoids synthesis are presented in Figure 14.
Synthesis of carotenoids, similarly to other substances, is characterized by a general pathway where a few carotenoids are the main precursors of others, that at the same time can act as intermediary or precursor for carotenoids with higher molecular complexity. The essential task in carotenoid synthesis is the construction of the characteristic polyene chain, that is, the hydrocarbon skeleton conjugated by alternating double and single bonds. Different reactions have been developed to build this basic chain with specifically located double and single bonds.

The first synthesized carotenoid was β-carotene, mainly because of its relative molecular simplicity and due to the fact that it is often a precursor of other carotenoids. Subsequently, the synthesis of different functional groups has led to the formation of an important variety of carotenoids. However, there is no general strategy for the direct and complete synthesis of carotenoids.

Consequently, the synthesis is based on a few general multistep pathways where various end groups are obtained from readily available key starting molecules. Therefore, although a great diversity of carotenoids can be synthesized under laboratory conditions, in practice less than ten of them are available at commercial scale, e.g. astaxanthin, astaxanthin-dimethyldisuccinate, β-carotene, canthaxanthin, β-apo-8'-carotenal, citranaxanthin, and the ethyl ester of β-apo-8'-carotenoic acid.

Carotenoids are spray-dried into stabilized matrix materials (antioxidants) and coated. They are usually formulated as 10% concentrated products and presented as a powder or beadlet for commercial use in animal nutrition.

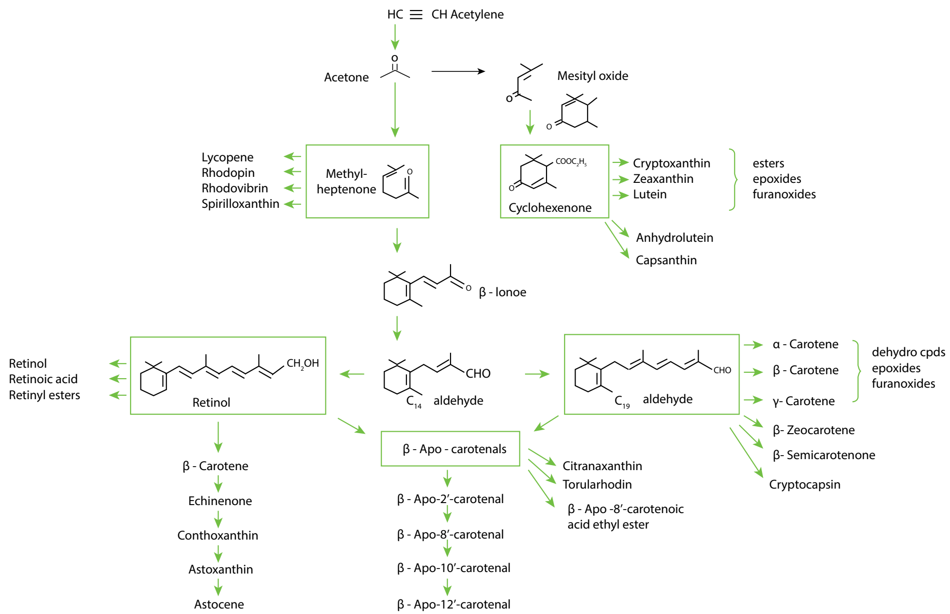


Figure 14 - *Chemicals schemes of carotenoid synthesis. [Kläui H., Bauernfeind J. C. 1981. Carotenoids as food colors. In: Carotenoids as Colorants and Vitamin A Precursors. Ed. J. C. Bauernfeind., Academic Press, New York, pp. 80.]*

3.2 Extraction from plants

Extracted substances derived from plants are also known as phytogenics or phytobiotics. Extraction procedure releases the carotenoids from the plant matrix and brings them into solution. Although most carotenoids in nature are found in plants, only Marigold flowers (Tagetes erecta), red peppers (Capsicum annuum – paprika) and annatto (Bixa orellana) seeds are used in practice for animal nutrition. Marigold flowers are one of the richest natural sources of lutein and zeaxanthin, whereas red peppers and annatto seeds are the most important sources of capsanthin and bixin, respectively.

The extraction of carotenoids involves the following stages:

- *Preparation of raw material*: while Marigold flowers are ensiled before extraction, red peppers are dried to minimize water content and ease the subsequent grinding. Some previous enzymatic and physical treatments can be used to improve the extraction. In the case of annatto seeds, the outer layers obtained by abrasion are used.
- *Extraction*: the obtained material is mixed with organic solvent(s) which is (are) the responsible for extracting the carotenoids, allowing the partitioning from the plant matrix. Grinding of Marigold and pepper optimizes the extraction by bringing the material in contact with the solvent. Parameters such as temperature and pressure are monitored. A crude extract of pigments with the solvent is obtained. The solvent is subsequently removed by distillation, obtaining an oleoresin extract of carotenoids. Due to its natural origin, minor amounts of other carotenoids can also be found mixed together with the main extract.
- *Saponification*: the carotenoids in the oleoresin are esters of fatty acids. Saponification allows de-esterifying the carotenoids to the free form, which is more easily absorbed, as well as removing the fatty acids and waxes. This process involves heating under an alkaline treatment.
- *Formulation*: the saponified extract is stabilized with antioxidant and the mixture is eventually added to a carrier.

3.3 Biosynthesis from micro-organisms

The use of microorganisms for the commercial biotechnological production of carotenoids is a promising route. For this purpose, certain microorganism strains are cultured in selected media and under specific conditions.

Microbial biosynthesis comprises the following common stages:

- *Cultivation*: microorganisms are grown in selected culture media and specific conditions. In the case of yeast and bacteria, fermentation in closed tanks with selectively enriched media occurs. In the case of algae, a variety of production systems are described, from outdoor ponds to closed tanks, with different levels of control over water properties (temperature, salinity, carbon dioxide, pH, etc). Irrespective of the microorganism cultured, conditions must support biomass production in a first stage, and subsequently trigger carotenoid accumulation by means of suitable changes in media conditions.
- *Microorganism harvesting*: microorganisms are filtrated or centrifuged to divide cells from their cultivation media. Cells are then killed by chemical or physical treatment. At this stage, cells obtained may be spray-dried to obtain a homogenous powder of dried killed cells, or can be subject to carotenoid extraction.
- *Carotenoid extraction*: cells are destroyed by chemical or physical methods to free the content of carotenoids. Carotenoids are then recovered by solvent extraction, and the solvent eliminated to obtain the purified pigment.
- Currently, there are several microorganisms with commercial potential for the production of carotenoid feed additives and

for example:

- *Phaffia rhodozyma (Xanthophyllomyces dendrorhous)* is a red yeast species, mainly cultured to produce astaxanthin, which is mostly obtained in its free and readily absorbable form.
- *Paracoccus carotinifaciens* is a red carotenoid-rich bacterium with predominance of astaxanthin, adonirubin and canthaxanthin.
- The fungus *Blakeslea trispora* is an active producer of ß-carotene.

Additionally, the unicellular freshwater alga Haematococcus pluvialis accumulates large amounts of astaxanthin. Most of the carotenoid is produced in the form of esters of fatty acids. Others with less relevance are the yeast Rhodotorula and the algae Chlorella and Dunaliella which produce β-carotene and astaxanthin.
Further developments in bioengineering to obtain hyperproducing strains as well as improvements in growth conditions and production processes may lead to optimized and more cost-effective carotenoids at a commercial scale.

3.4 Technological aspects of use in feed

a. End product forms

Carotenoids produced on a commercial scale are normally formulated for ease of use, to standardize their concentration and protect them. The end presentation of the commercial product has also implications in the long-term quality of the carotenoids. Depending on the carrier and technological treatments, different forms are available on the market:

- Liquid: the carrier is water. Carotenoids must be present in their free (unesterified) form to be water-soluble.
- Solid: the end product must be free flowing to ensure suitable dosing in feed by preventing the occurrence of caking and lumping. Two main presentations are found on the market:

  - Powder: using different powder substances, mainly anticaking agents (e.g. silica) and feed materials (e.g. calcium carbonate). In the case of carotenoids extracted from microorganisms, carotenoid-rich cells can be spray-dried thereby becoming the carrier itself; in such a case different treatments may be needed to optimize the bioavailability and content standardization of carotenoids in the end product.
  - Beadlets: the carotenoid is distributed in a starch-encapsulated gelatine and/or carbohydrate matrix, with the addition of antioxidants.

These liquid and solid formulations are developed to ensure that the carotenoids are stable, easily mixable, and are homogeneously distributed in premixture, feed and water. The choice of the most appropriate product presentation will depend on different factors such as the intended uses, price or expected shelf-life. Products are adjusted to a certain carotenoid content, according to technical specifications of the product.

b. Homogeneity

The process of including the carotenoid in a liquid or solid carrier is performed in a way to ensure that the carotenoid and the carrier are homogenously mixed. Optimum homogeneity is regularly evaluated by taking several samples at different times during the packaging of a single produced batch. Homogeneity is ensured when the amount of pigment per unit of product weight is the same in all the samples and is within the range indicated in the product technical specifications.
Appropriate homogeneity is crucial to guarantee the accurate dosage and homogeneous distribution of the carotenoid in the compound feed offered to the animals.

c. Stability

Depending on their structure, carotenoids are generally extremely unstable compounds which are subject to a variety of rapid oxidative degradation processes. For example, in feed materials such as cereals, alfalfa and other green plants, carotenoids are not usually stable. Depending on the source, their pigmenting effect may decrease with the maturation process of the crop; it may also decrease as a result of particular processing methods, storage conditions, length of storage, composition of the feed or other, less well documented causes. The variations in the carotenoid content of raw materials in feeds are great, and the losses incurred during processing and storage are in some cases considerable and difficult to predict. As a result, traditional pigmenting material appears to be of only limited suitability as a source of adequate and consistent pigmentation, and has to be constantly checked.

Furthermore, it is also difficult to control the pigmenting properties of compound feed because of the great differences in the quality of the raw material components. The carotenoid content of natural feedstuffs is not constant and can vary greatly according to the type of raw material, its origin, the cultivation method, climate, manuring and harvesting. Because of this, supplemental carotenoid products are largely preferred in practice in order to achieve a consistent degree of pigmentation.

As reviewed in other chapters, carotenoids have also been attributed important antioxidant properties. An antioxidant prevents the oxidation of other substances by oxidizing itself. Thus, carotenoids are easily oxidized and therefore degraded, which affects the pigmenting performance. Consequently, carotenoids must be protected to guarantee the quality throughout the shelf-life of the commercial product as well as in the pre-mixtures and in the compound feed.

Carotenoids are mainly protected from oxidation by 3 methods:

- Addition of antioxidants, e.g. ethoxyquin or tocopherols.
- Micronisation to create coated beadlets or micropearls. In this case, this method is combined with the use of antioxidants in the matrix of the beadlets.
- Coating.

3.5 Commercial products

a. Nature identical carotenoids prepared by total synthesis

Carotenoids	Astaxanthin, astaxanthin-dimethyldisuccinate, canthaxanthin, citranaxanthin, β-apo-8’-carotenoic acid ethyl ester (apoester), β-apo-8’-carotenal.
Commercial forms	Beadlets or powders with antioxidants, carbohydrates and/or gelatine
Carotenoid content	Usually 10%.

b. Extraction from plants

Carotenoids	Combination of carotenoids with predominance of lutein/zeaxanthin, capsanthin/capsorubin or bixin in accordance to the different plant extracts
Commercial forms	Powders with antioxidants and inert anticaking agents. Liquid by being diluted in water
Carotenoid content	Usually 1 to 10% depending on technical specifications

c. Biosynthesis from micro-organisms

Carotenoids	Combination of carotenoids rich in astaxanthin (yeast Phaffia rhodozyma); combinantion of red carotenoids rich in astaxanthin, adonirubin and canthaxanthin (Paracoccus carotinifaciens).
Commercial forms	Extracted carotenoids or dried killed cells with antioxidants, and/or carriers to standardize the products
Carotenoid content	*P. rhodozyma:* >10% *P. carotinifaciens:* astaxanthin (>2%), adonirubin (>0.7%), canthaxanthin (>1%)

4. CONDITIONS OF USE OF CAROTENOIDS

Main carotenoids used in animal nutrition include, astaxanthin, β-carotene, β-apo-8'-carotenoic acid ethyl ester, lutein, zeaxanthin, canthaxanthin and capsanthin. These are approved feed additives for animal nutrition in the European Union and most of the countries around the world. These additives are foreseen for use in feed and/or water for different animal species.

According to its main indications these additives are classified in the EU as carotenoids showing the following functions:

- Substances that add or restore colour in feedstuff;
- Substances which, when fed to animals, add colours to food of animal origin;
- Substances which favourably affect the colour of ornamental fish or birds.

Nevertheless, in some animal species carotenoids are also used for other purposes, such as to improve the immune and reproductive status.

It is important to differentiate the concepts of pigmentation and colouration, which are usually confused with each other. However, although related, they are different and it is necessary to make a distinction. Both concepts have a different interpretation and are evaluated in different ways. Pigmentation is evaluated by analytical chemistry, normally using HPLC, to quantify the concentration of carotenoids retained in tissues (e.g. egg yolk, skin, flesh) and is expressed in mg of carotenoid per kg of tissue (mg/kg or parts per million (ppm)). Near Infrared Reflectance Spectroscopy can also be used to estimate the level of carotenoids in the tissues.
Colouration is commonly measured be either using Colour Fans (Fig 14, 15 and 16), or by a colourimeter which is an instrumental method of measuring the redness, yellowness and brightness of the tissues. While more subjective than colourimetric methods, Colour Fans are

the most practical and economical method, and are widely use for the colour assessment of food products (e.g. egg yolk, broiler tissues and salmonids flesh). It is important to clarify that a certain amount of carotenoid deposited in the food products always have the potential to express a certain colour. However, to attain a determined colour, it is necessary to take into account several factors that affect the conditions of use of the carotenoids.

Furthermore, the level of use of carotenoids as feed additives is conditioned by:

- The quantity of total carotenoids necessary to achieve the proper colour intensity of the food products. Intensity of a colour depends of the consumer demand within a market.
- Total pigmentation costs to achieve the desired colour score.

Usually, there are no limitations regarding the animal age or production stage for carotenoids use, although some constraints may exist in some countries. Overall, there is no withdrawal period and there are no contraindications. Nevertheless in some countries, such as the EU, maximum limits in complete feedstuffs are established.

Overall, optimal pigmentation and colouration depend greatly on the amount of carotenoids added to the feed. In general the amount of additive is dependent on the basic pigment content in the raw materials and the desired pigmentation. Since a particular pigment utilization is dependent on many variable factors, and therefore cannot be precisely predicted, there are no general rules governing supplementation, only specific guidelines based on years of scientific trials and experience. The general conditions of use for some of these carotenoids for different animal species are described below. In addition, each supplier may have its own recommendations.

4.1 Poultry

There are two components of egg yolk and broiler pigmentation. The first (referred to as the saturation phase) involves the deposition of yellow carotenoids to create a yellow base. Such a yellow base is very important for good saturation of the final colour. Once the yellow base is established, the addition of the red carotenoid changes the colour hue to a more orange-red colour (the second component referred to as the colour phase). The dose-related colour response to red carotenoids is higher than the response to yellow carotenoids, and the combination of yellow and red carotenoids is therefore more cost effective for pigmentation. Figure 15 shows this principle for egg yolk pigmentation.

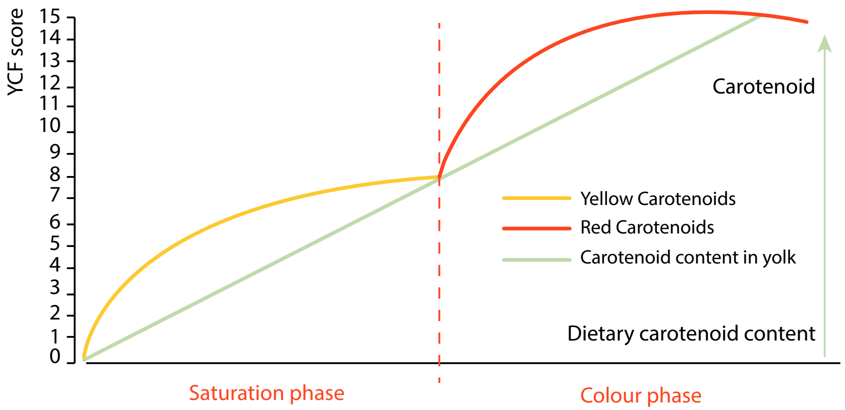


Figure 15 - *Egg yolk pigmentation phases.*

a. Laying hens and other layer species

Carotenoids are used in laying hens to enhance the colour of egg yolk (Figure 16). To this end, yellow pigments alone or in combination with red carotenoids can be used, according to the colour and hue desired by the market, where colour measure can be done using the Yolk Colour Fan (scale from 1-15) (Figure 17).

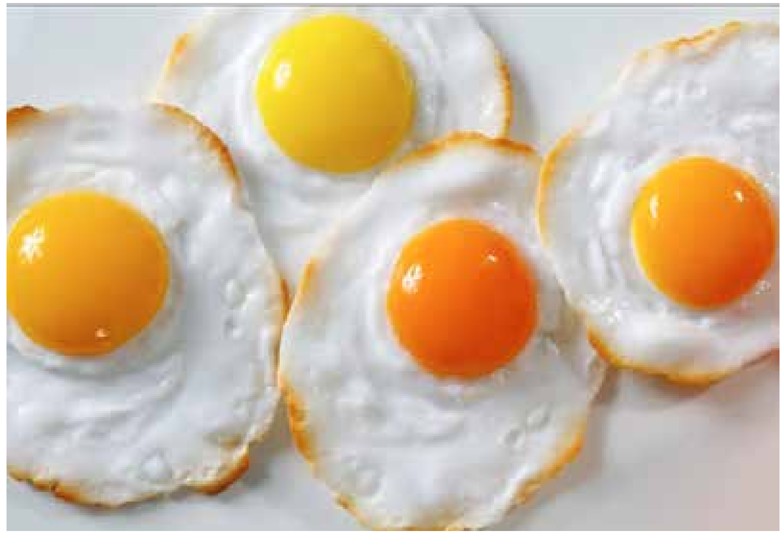


Figure 16 - *Comparative yolk pigmentation.*

Figure 17 - *Yolk Colour Fan.*

Commercial xanthophyll sources typically used to achieve the desired pigmentation and colour include: (yellow sources) lutein, zeaxanthin and the β-apo-8'-carotenoic acid ethyl ester (apoester) and (red sources) capsanthin, citranaxanthin or canthaxanthin. The blending of different yellow and red xanthophylls regardless of the sources (cfr Chapter 4) is the most appropriate and economical option to yield yolk colour according to different market needs.
When formulating supplemental carotenoids, however, the pigmenting contribution of native xanthophylls, supplied by different feedstuffs (e.g. yellow maize, alfalfa, gluten meal, etc.), must also be taken into account. This allows to better determine the right level of supplemental carotenoids in feed. Formulation should also give consideration to the relative deposition of different carotenoids in yolk, as it is directly linked to their pigmenting efficacy. The same principle, of pre-checking native xanthophylls, also applies when pigmenting broiler skin.

Because pigmentation is affected by several factors, in some particular cases higher doses of xanthophylls can be necessary to achieve the desired colour values. After supplementation, intensification of yolk colour becomes apparent at about 48 hours after ingestion of the carotenoid. However in order to obtain uniform and maximum deposition in the yolk, the laying period needs to be preceded by 9-11 days of supplementation.

Two examples of carotenoid recommendations, according to target Yolk Colour Fan scores are presented in Table 3 and Table 4.

Table 3 - *Example of Yolk Colour Fan score (YCF) obtained in egg yolk, combining lutein (tagetes extract) with capsanthin (paprika extract) in diets for laying hens.*

Step 1: Calculate the xanthophyll content of the feed				Step 2: Adjust supplementation based on target Colour Fan Score		
Feed Ingredient	Yellow Xanthophyll content (ppm)*	Ingredient Inclusion rate in feed (%)	Total Xanthophylls (ppm)	Yellow carotenoids in Feedstuffs (ppm)	Supplementary Xanthophylls (active substance)	Recommended levels of active substance (mg/kg feed) needed to achieve a YF score of 11-12**
Yellow corn	14	20	2.8			
Other Ingredients	0	80	0			
Total Yellow carotenoids in feedstuffs			2.8	2.8	Yellow (lutein - tagetes extract) Red (capsanthin - paprika extract)	2.0 - 6.0 2.9 — 4.9

* Xanthophyll content of feedstuffs varies depending on strain differences, harvest, processing and storage conditions
** Values referred to the standard Yolk Color Fan with colour grades 1 to 15. – Based on daily feed consumption of 110g/hen /day

Table 4 - *Example of Yolk Colour Fan score (YCF) obtained in egg yolk, combining apoester with canthaxanthin in diets for laying hens.*

Step 1: Calculate the xanthophyll content of the feed				Step 2: Adjust supplementation based on target Colour Fan Score		
Feed Ingredient	Yellow Xanthophyll content (ppm)*	Ingredient Inclusion rate in feed (%)	Total Xanthophylls (ppm)	Yellow carotenoids in Feedstuffs (ppm)	Supplementary Xanthophylls (active substance)	Recommended levels of active substance (mg/kg feed) needed to achieve a YF score of 11-12**
Yellow corn	14	20	2.8			
Other Ingredients	0	80	0			
Total Yellow carotenoids in feedstuffs			2.8	2.8	Yellow (apoester) Red (canthaxanthin)	2.0 - 2.5 2.5 - 3.0

* Xanthophyll content of feedstuffs varies depending on strain differences, harvest, processing and storage conditions
** Values referred to the standard Yolk Color Fan with colour grades 1 to 15. – Based on daily feed consumption of 110g/hen /day

b. Poultry meat

In a similar way to layers, carotenoids are used in broilers (Figure 18) and other meat poultry species to confer the desired yellow-orange hue of skin, shanks, peak, toes and fatty tissues of these animals, where colour measure can be done using the Broiler Colour Fan (scale from 101-108) (Figure 19).

Figure 18 - *Comparative broiler carcass pigmentation.*

Figure 19 - *Broiler Colour Fan.*

Once again, xanthophylls can be used alone or in combination depending on the colour and hue desired by the market. Combination of yellow and red carotenoids is the most appropriate and economical solution to deliver the targeted colour. Also here, endogenous xanthophylls supplied by different feed ingredients (e.g. yellow maize, alfalfa, gluten meal, etc.) must be taken into account.
As pigmentation is affected by several factors, in some particular cases higher doses of xanthophylls can be necessary to achieve the assigned colour values. Since lipid metabolism is slow in broilers during the high growth period, and pigments are deposited in non-specific tissues (skin, shank, etc), higher dosages are required for pigmentation compared to the laying animals. Red xanthophylls are necessary to give a golden colour.
An increase in colour intensity of the skin after five days of supplementation has been reported. Best results are usually achieved when feeding the carotenoid supplemented diet during the last three to four weeks before slaughter.
With regards to poultry carcass, the colour is linked to specific cultural habits. In particular, in the South European countries, yellow/orange

chickens are preferred, due to the history of use of feed materials rich in carotenoids (such as yellow maize) in the past. In the Northern European countries however, the less pigmented chickens are usually preferred.
Only yellow birds (depending on genetic) can deposit the carotenoids in fat and skin. Most white birds are unable to store and thus it is impossible to pigment their tissues. Carotenoids can also be deposited in the shanks of certain poultry (dependent on their strain). In such a case, coloured legs are perceived by the consumers as a sign of bird's good bird health and high quality.
Examples of the use of yellow pigments in combination with red pigments are illustrated in Table 5 and 6

Table 5 - *Example of Broiler colour obtained in skin, combining lutein (tagetes extract) with capsanthin (paprika extract) in diets for Broilers.*

Step 1: Calculate the xanthophyll content of the feed				Step 2: Adjust supplementation based on target Colour Fan Score		
Feed Ingredient	Yellow Xanthophyll content (ppm)*	Ingredient Inclusion rate in feed (%)	Total Xanthophylls (ppm)	Yellow carotenoids in Feedstuffs (ppm)	Supplementary Xanthophylls (active substance)	Recommended levels of active substance (mg/kg feed) needed to achieve a YF score of 11-12**
Yellow corn	14	25	3.5	3.5 →	Yellow (lutein - tagetes extract) Red (capsanthin - paprika extract)	34 – 40 5.75 - 11.40
Other Ingredients	0	75	0			
Total Yellow carotenoids in feedstuffs			3.5			

* Xanthophyll content of feedstuffs varies depending on strain differences, harvest, processing and storage conditions
** Categorisation on low, medium and high pigmentation level according to standard market preferences. Following supplementation from 21 days of age.

Table 6 - *Example of Broiler Colour Fan Score (BCF) obtained in skin, combining apoester with canthaxanthin in diets for Broilers.*

Step 1: Calculate the xanthophyll content of the feed				Step 2: Adjust supplementation based on target Colour Fan Score		
Feed Ingredient	Yellow Xanthophyll content (ppm)*	Ingredient Inclusion rate in feed (%)	Total Xanthophylls (ppm)	Yellow carotenoids in Feedstuffs (ppm)	Supplementary Xanthophylls (active substance)	Recommended levels of active substance (mg/kg feed) needed to achieve a YF score of 11-12**
Yellow corn	14	25	3.5	3.5 →	Yellow (apoester) Red (canthaxanthin)	16.5 – 25.5 4.0 - 5.0
Other Ingredients	0	75	0			
Total Yellow carotenoids in feedstuffs			3.5			

* Xanthophyll content of feedstuffs varies depending on strain differences, harvest, processing and storage conditions
** Categorisation on low, medium and high pigmentation level according to standard market preferences. Following supplementation from 21 days of age.

4.2 Fish

In the case of salmonids (e.g. salmon and trout) the aim of using red oxycarotenoids in their diets is to enhance the orange-pink colour of the flesh (Figure 20). In the case of other fish species (e.g. Red Tilapia, Red Seabream, Ornamental), skin pigmentation is the target.

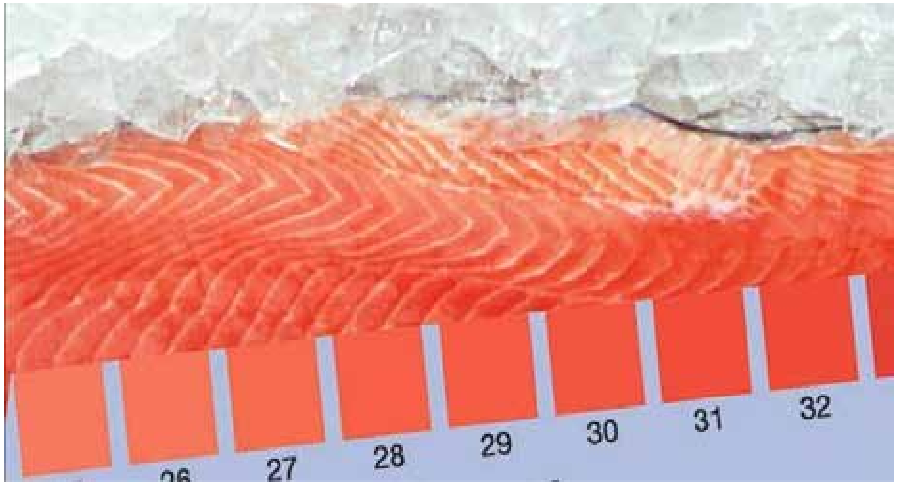


Figure 20 - *Measurement of salmon flesh pigmentation.*

For flesh pigmentation, the carotenoid most commonly used is astaxanthin, although in some markets it can be combined with canthaxanthin. These carotenoids are normally supplemented to fish from the age of six months onwards.
Every farming company should decide upon a pigmentation strategy to give the required colour to the fish at harvest. Flesh colour is one of the most important quality criteria for salmonids. The flesh colour requirements vary between different markets and therefore the farmer must design a pigmentation strategy that optimises final flesh colour suitable for each target market.
In terms of dosing, approximately 60 mg of astaxanthin per kg finished feed is the most efficient dietary dose for optimal pigment re-

tention in Atlantic salmon, while in freshwater rainbow trout the level has been reported to range from 50-70 mg/kg astaxanthin. There is a large range of pigment retention rates between species. Literature data and field information suggest the following values for each species. Rainbow Trout: 20-25%, Coho Salmon: 14-18% and Atlantic Salmon: 8-10%. As a general guide, Table 7 presents the minimum estimated flesh astaxanthin concentrations required to attain a certain fillet colour. For Tilapias' skin pigmentation, some fish farmers are also using capsanthin.

Table 7 - *Minimum estimated flesh astaxanthin concentrations required to attain a certain fillet colour.*

Species	Colour[1]	Concentration of astaxanthin in the muscle (mg/kg)2
Coho Salmon	30-32	16-18
Rainbow Trout	31-33	20-22
Atlantic Salmon	29-31	8-10

[1] Colour measured by Salmon Colour Fan (scale from 20-34) - 2 Values obtained by field measurements in Chilean salmon

4.3 Crustaceans

In crustaceans, carotenoids are used to enhance the pigmentation of the exoskeleton. Similarly to fish, the carotenoid most commonly used is astaxanthin, but also β-carotene is used, although in some markets it is combined with canthaxanthin. In crustaceans, astaxanthin is deposited below the cuticle, over the flesh.
Astaxanthin is commonly used at between 4.0 and 80.0 mg/kg of compound feed, depending on the desired intensity of pigmentation required. It is important to know the concentration of the carotenoid product when calculating the dosage requirements. Astaxanthin is also used during the larval stages, to enhance immunity and to increase survival rates. In this case, doses are dependent on the re-

search and know-how developed by the industry. Some farmers also use capsanthin for shrimp pigmentation.

4.4 Ornamental birds

Feed for ornamental birds, mainly canaries is supplemented with β-carotene, canthaxanthin and lutein with a view to colour them. Supplying these carotenoids to birds gives a bright orange-red colour to their feathers.
Canthaxanthin and β-carotene are specifically used in colour feeding Red Factor Colour Bred Canaries to maintain their rich red plumage. In order to obtain bright red feathers, a ratio of 1:1 of canthaxanthin and β-carotene is required.

4.5 Dogs and cats

In dogs and cats lycopene, β-carotene, lutein and zeaxanthin are usually used for other purposes than pigmention. They are usually used in feed, alone or in combination, as antioxidant source, to enhance immune status, as well as to prevent age-related macular degeneration of the eye. In fact, commercial feeds aimed at geriatric pets are frequently enriched with lutein-zeaxanthin for such a purpose. Doses and conditions of use of lutein and zeaxanthin in this context are very much depending on the research and know-how developed by the industry.

5. SAFETY OF USE

As feed additives, the carotenoids are regularly evaluated by regulatory agencies such as the European Food Safety Authority (EFSA in EU) or the Food and Drug Administration (FDA in USA) for their safety along the feed and food chain. The evaluations lead to the publication of scientific opinions or regulatory decisions providing information on the basis for the evaluation and concluding on the safety of the carotenoids under their proposed conditions of use in animal nutrition.
Furthermore, some of the carotenoids are also used in human nutrition and for this reason are evaluated either within the European Union by other panels from EFSA or at global level, in particular by the Joint Expert Committee on Food Additive of the World Health Organisation and of the Food and Agriculture Organisation (WHO/FAO JECFA). This also leads to the publication of scientific opinions providing the results of the evaluation of the Panel of experts.
When safety of carotenoids is evaluated for their use in animal nutrition, the safety assessment covers the whole feed and food chain, i.e. the safety for the target species, the safety for the consumer of the animal product, the safety of the workers handling the carotenoids during the manufacture of feeds and the safety for the environment, as such and after feeding to the animals. In Europe, the evaluations carried out by EFSA on the use of Carotenoids in Animal Nutrition are publically available at: http://www.efsa.europa.eu/en/panels/feedap.htm.

5.1 Safety for the target species

The safety for the target species is evaluated on the basis of trials made on the target animal species. These trials aim at overdosing the target animal compared to the proposed conditions of use in order to evaluate the possible negative effect on the animals of these high dosages and thus determine a safety margin between the proposed conditions of use and the dose with an effect. When evaluating the effect, parameters such as performance, blood analysis and possible impact on organs at low safety margins are verified.

The recent evaluations of the different carotenoids over the last few years have demonstrated that carotenoids are generally well tolerated by the target animal species, either poultry or salmonids as well as pet animals, ornamental birds and fish. In addition, carotenoids have been used for decades in animal nutrition with no reports of negative effects on animal performance and health.
As mentioned earlier, some carotenoids are used both in feed and in food. In such case, the manufacturer of the carotenoids may base its safety evaluation for the target animal on the safety for the human consumer, in particular, when the same dose is recommended.

5.2 Safety for the consumers

The safety assessment of carotenoids for the consumer of animal products containing the carotenoids, when used in animal nutrition at recommended dose is performed in two steps:

- the hazard assessment
- the consumer exposure assessment

The hazard assessment aims at defining the potential effect of the carotenoids for the consumers. This hazard assessment is mainly based on studies run on laboratory animals (rats and mice), where the effect of increasing dose of carotenoids is measured. The parameters vary between the carotenoids and usually cover the effect on health, the impact on organ and the potential for mutagenicity and carcinogenicity. Series of trials are usually run in accordance with globally harmonised standards (OECD) and lead to the setting of a Non Observable Adverse Effect Level (NOAEL). The NOAEL is the highest tested dose that has shown no adverse effect on the animals. At higher doses, adverse effects would be seen on the animals. In some case, the tests may show that a NOAEL cannot be defined. This means that no adverse effects were seen at any levels tested.
When a NOAEL is set, the assessors usually apply a safety margin of 100 to calculate the Acceptable Daily Intake (ADI) for the consumer. The safety margin takes into account the intra-species variability (variability of the human population with regards to the effect – factor = 10) and the inter-species variability (a margin of safety that takes into account that the effect was evaluated on laboratory animals – factor =10):

Safety margin (100) = intra-species variability (10) x inter-species variability (10)

The ADI is defined as the dosage of the carotenoids that could be consumed by the humans daily during his/her whole lifetime without an adverse effect. Any intake below this level is considered as safe. Furthermore, as the ADI refers to the whole lifetime of the consumer, excursion during short period of time above the ADI are also considered as safe. This means that if a consumer would consume more than the ADI for a short period of time, this might not impact his/her health, as soon as the total amount during the lifetime is below the ADI. The ADI is expressed in mg / kg bodyweight and is calculated as follows:

$$\text{Acceptable Daily Intake (ADI)} = \frac{\text{Non Observable Adverse Effect Level (NOAEL)}}{\text{Safety margin (100)}}$$

The consumer exposure assessment aims at calculating the potential intake of the carotenoid by the consumer, based on carotenoid concentration in the food of animal origin and on the estimated consumption of that food. Different scenarios are used to evaluate the consumption of food, from very conservative evaluation (the food basket considering that consumers are eating every day, 300 g of meat or fish, 100g of eggs,…) to evaluation based on detailed consumption survey.

In general, the consumer exposure assessment provides for additional margin of safety. The consumer exposure is calculated as follows:

Consumer Exposure (CE) = (Food concentration x Food consumption)

The consumer safety is then the comparison between the consumer exposure (CE) and the acceptable daily intake (ADI) as follows:

- If CE > ADI, the proposed conditions of use are not acceptable
- If CE <= ADI, the proposed conditions of use are acceptable.

It should be noted that, when no NOAEL has been set following the evaluation of the tests, no ADI can be derived and hence there is no concern with the safety of the related carotenoids. In that case the evaluation concludes 'ADI not specified'.
In some cases, the existing tests do not allow conclusions on the NOAEL and when this occurs no ADI can be set. (In this case, the evaluation concludes 'no ADI allocated').
The carotenoids have been evaluated by different committees and Panels and have led to the setting of ADI for some carotenoids (canthaxanthin, lutein and zeaxanthin). Over the next few years, it is expected that carotenoids will continuously be reviewed and ADI values will be further updated.
In certain markets, a maximum limit is included in the conditions of the use (set up in the authorising regulation) of the carotenoid.

5.3 Safety for the workers

The safety for workers is also based on two steps:

- the hazard assessment;
- the worker exposure at work place assessment.

The hazard assessment is based on laboratory animal studies, linked to other routes of contact other than food consumption, i.e. inhalation test and test on the eyes and skin. Up till now, the animal tests that have been run on carotenoids have demonstrated that the hazard for the worker is low by inhalation or contact to skin and eyes. The workers exposure takes into account the way the product is handled and also its forms. Carotenoids are usually placed on the market as a preparation formulated for protecting the carotenoids from oxidation. These formulations also aim at reducing the risk for the workers through controlled exposure to dust (e.g. low particle size, or dusting potential).

5.4 Safety for the environment

As indicated in the former chapters, carotenoids are present in nature and are oxidised quite readily. Therefore, the potential direct exposure to the environment with the carotenoids is considered as low and no negative environmental impact is foreseen with regards to their handling in the feed chain and when used in animal nutrition.

6. CAROTENOIDS’ MONOGRAPHS

6.1 Ethyl ester of β-apo-8’-carotenoic acid

Chemical Formula	$C_{32}H_{44}O_2$
Molecular weight	460.70 g / mol
Synonyms	8’-apo-beta-carotenoic acid ethyl ester Apocarotenoic acid ethyl ester Apocarotenoic ester Apoester
Chemical name	Ethyl-8’-apo-beta-caroten-8’-oate Ethyl-all-trans-2,6,11,15-tetramethyl-17-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8,10,12,14,16-heptadecaoctaenoate
CAS Number	1109-11-1
Physical parameters	Apo-carotene ester is normally used in animal nutrition as a preparation. The preparations are brown-red free flowing powder, usually containing 10 % of apo-carotene ester.
GHS classification	Not classified a according to the GHS classification. The Classification of the preparations depends on their composition.
Analytical methods	FEFANA 17.10.2008. Determination of β-Apo-8’-carotenoic acid ethyl ester (Apoester) by Photometry in the Maximum at approx. 446 nm FEFANA 20. 07. 2007. Determination of Apoester in Premixes, Feedstuffs and Water by High Performance Liquid Chromatography (HPLC)[1]

[1]Available upon request to FEFANA

6.2 Astaxanthin

Chemical Formula	$C_{40}H_{52}O_4$
Molecular weight	596.85 g/mol
Synonyms	3,3’-Dihydroxy-[all-trans-1,18-(3,7,12,16-tetramethyl-1,3,5,7,9,11,13,15,17-octa-decanonaen-1,18-diyl)-bis-(2,6,6-trimethylcyclohexen)]-4,4’-dione (3S,3’S:3R,3’R:3R,3’S = 1:1:2) ’rac’-Astaxanthin
Chemical name	3,3’-Dihydroxy-beta,beta-carotene-4,4’dione
CAS Number	7542-45-2
Physical parameters	Violet-brown to violet-red free flowing 10% formulation.
GHS classification	Not classified according to the GHS classification. The Classification of the preparations depends on their composition.
Analytical methods	FEFANA 30.05.2008, Determination of Astaxanthin tel-quel by Photometry at the isobestic wavelength of 431 nm; FEFANA 2007.07.20. Determination of stabilised asthaxanthin in Premixes and Feedstuffs by High Performance Liquid Chromatography (HPLC)[2]

[2] Available upon request to FEFANA

6.3 Canthaxanthin

Chemical Formula	$C_{40}H_{52}O_2$
Molecular weight	564.82 g / mol
Synonyms	Canthaxanthin
Chemical name	Beta-carotene-4,4'-dione 4,4'-diketo-beta-carotene
CAS Number	514-78-3
Physical parameters	Canthaxanthin is normally used in animal nutrition as a preparation. The preparations are violet-red to red-violet free flowing powder, usually containing 10 % of canthaxanthin.
GHS classification	Canthaxanthin is classified as "aquatic chronic 3" according to the GHS classification. The Classification of the preparations depends on their composition.
Analytical methods	FEFANA 30.05.2008. Determination of Canthaxanthin tel quel by Photometry at the Isobestic Wavelength of 426 nm FEFANA 01.12.2010. Determination of Canthaxanthin in Premixes, Feedstuffs and Water by High Performance Liquid Chromatography (HPLC)[3]

6.4 Capsanthin

Chemical Formula	$C_{40}H_{56}O_3$ (as capsanthin)
Molecular weight	584.87 (as capsanthin)
Synonyms	Paprika extract; Paprika oleoresin; Capsanthin/Capsorubin
Chemical name	(3R,3'S,5'R)-3,3'-Dihydroxy-beta,kappa-caroten-6'-one (as capsanthin)
CAS Number	465-42-9 (as capsanthin)
Physical parameters	Active substance: Dark reddish viscous paste with a characteristic paprika odour. Feed additive preparations: Orange to reddish free flowing powder containing different concentrations of the active substance.
GHS classification	Not classified according to the GHS classification. The Classification of the preparations depends on their composition.
Analytical methods	FEFANA 01.10.2010, Determination of Lutein, Zeaxanthin and Capsanthin telquel, in premixes and in feed, by HPLC at 450 nm wavelength.

[3] Available upon request to FEFANA

6.5 Lutein

Chemical Formula	$C_{40}H_{56}O_2$ (as lutein)
Molecular weight	568.88 (as lutein)
Synonyms	Tagetes extract; Tagetes oleoresin; Vegetable lutein
Chemical name	(3R,3'S,6'R)-beta,epsilon-Carotene-3,3'-diol (as lutein)
CAS Number	127-40-2 (as lutein)
Physical parameters	Active substance: Deep orange to reddish viscous paste. Feed additive preparations: Deep orange free flowing powder or liquid containing different concentrations of the active substance.
GHS classification	Not classified according to the GHS classification. The Classification of the preparations depends on their composition.
Analytical methods	FEFANA 01.10.2010, Determination of Lutein, Zeaxanthin and Capsanthin telquel, in premixes and in feed, by HPLC at 450 nm wavelength.

7. BENEFITS FOR CONSUMERS AND FOOD PRODUCTS

Humans experience nature in all its coloured splendour through their eyes. Life without colour is unthinkable. Colours are an integral part of man's daily experience. No wonder, colours also have an important function in cooking. Humans also "eat" with their eyes. It is considered one of man's basic experiences that a particular food has to be of a particular colour in order to be edible. Food triggers anticipatory physiological and psychological reactions conditioned by experience, tradition, education and environment. Natural, fresh colours whet the appetite, enhance enjoyment of food and also act as a kind of optical seasoning. As a rule, consumers prefer products with more intensive colouration.

By contrast, unnatural-looking colours give rise to mistrust and rejection. Of the different quality criteria, colour is therefore one of the most important, though frequently underestimated. Thus it is no coincidence that berry and plant extracts such as saffron, paprika, annatto, carrots, peppers, etc. have been used for centuries to refine foods everywhere. In addition, there is a connection between colour and health. In plants as in animals, deficient pigmentation and paleness are often the first signs of malnutrition or disease. It is therefore no surprise that there is normally an instinctive rejection of foods of plant or animal origin which appear unusually pale. The impression of colourlessness is associated, often unconsciously, with ill health or poor quality. Natural, bright colours, on the other hand, give the impression of high-quality, healthy, nutritious food. This is especially true of the usually rich natural pigmentation of egg yolk, the epidermal and subepidermal tissue of poultry, the flesh of salmonids, and the shells and epidermis of most crustaceans. It is particularly in such products that, in addition to other criteria, consumers appreciate and show a preference for the more intensive colouration.

Carotenoids are widely distributed natural pigments, and as such are well suited to meeting the growing demand from large groups of consumers for the use of only naturally-occurring pigments in feed and food products.

The provision of an adequate supply of carotenoids is also of considerable interest from the viewpoint of nutritional science. Poultry farming and aquaculture, for instance, are unthinkable without adequate quantities of carotenoids as feed additives. In this context, the carotenoids perform essential biological functions and make an important contribution to the improvement of pigmentation, thus raising the quality as well as the market value of the product in question. Although in this respect, in principle, the most diverse additional pigment sources are available, many of them are quite unsuitable for use as effective pigments in animal production.

The benefits for the consumers are visible at three levels:

- The colour provided by the use of carotenoids in feed is a quality sign for most of the consumers;
- The colour of the poultry products linked to the use of carotenoids in animal nutrition provides an indication of the quality and hygiene of the reared poultry (see importance of carotenoids for health);
- The presence of carotenoids in animal products is a good source of biological antioxidants for the consumers, in addition to the carotenoids found in food from plant origin.

Carotenoids used in feed are deposited in fatty tissues, in particular in the egg yolk and in the subcutaneous fat of animals, and in the case of fish in the muscles or skin, and in the cuticle in case of crustaceans. Due to their colour characteristics (hue and lightness), carotenoids provide colours to these products:

- In the egg yolk, as the carotenoids are concentrated, the amount of carotenoids necessary to get an appealing colour is reduced.
- In the food products containing eggs, in order to colour the food product, the quantity of carotenoids to be used in the feed depends on the level of colour and the number of eggs used in the end product. Usually, the concentration of carot-

enoids in the eggs yolk for this application should be higher.
- In the poultry carcass, the carotenoids are diluted all over the subcutaneous fat, hence higher concentration of carotenoids in feeds is necessary to achieve the appropriate level of carotenoids in the fat.
- Pink colour of flesh of the Salmonids,
- In Crustaceans, it gives the typical colour of the cuticle once cooked.

Market studies have demonstrated that homogeneously coloured egg yolks were preferred by the consumers. This is usually related to the good health status of the animals.

Apart from allowing improving the aspect of food and making it more appealing according to the consumer preferences, the presence of carotenoids in food offers a variety of benefits others than colouring:

a. Lutein-Zeaxanthin

We refer to lutein and its stereoisomer zeaxanthin altogether, as they are the major components in the Marigold flower extract, which is the main source of these two carotenoids.
In humans, the lutein and zeaxanthin are believed to function in two ways: firstly as an antioxidant, thereby protecting from oxidative damage, and secondly as a filter of ultraviolet light. Evidence indicates that the consumption of lutein and zeaxanthin is related to a lower incidence of age-related macular degeneration (AMD) and cataracts. This is supported by the fact that lutein and zeaxanthin are the only carotenoids deposited in the lens and the macula lute, an area of the retina responsible for central and high acuity vision. In fact, a number of human studies have demonstrated that supplementing these carotenoids increases the macular pigment and improves the vision in patients with AMD and other ocular diseases.
Lutein may also serve to protect skin from UV-induced damage

and may reduce the risk of cardiovascular disease. Moreover, and mostly linked to their antioxidant properties, there is strong epidemiologic evidence that lutein can protect against the development of certain types of cancer. Additionally, and similarly to what it has been observed in different animals, lutein has been indicated to improve the immune response.
Today, lutein and zeaxanthin are added to the diet by means of both supplements and functional foods, the latter being purposely enriched with these carotenoids. An excelling case of functional food is eggs enriched with xanthophylls. In this respect, it has been consistently proved that adding lutein and zeaxanthin to the diet of laying hens allows greatly increasing the content of these carotenoids in the egg's yolk, in a form which becomes a highly digestible and bioavailable dietary source for humans. Thus, in addition to different green leaved vegetables, lutein-zeaxanthin enriched eggs constitute an ideal source of these natural antioxidants for consumers.

b. Astaxanthin

Similarly to the effects described for lutein, a positive role in many human health problems has been associated to the consumption of astaxanthin, such as UV-light protection and anti-inflammatory properties, mainly linked to its antioxidant power. There are also positive human clinical trials supporting eye health, showing that astaxanthin helps diabetic retinopathy, macular degeneration, eye strain and fatigue and seeing in fine detail.
Astaxanthin has shown immune-boosting effects in humans, improving the ability of protective white blood cells to surround and destroy infecting organisms, especially fungi such as Candida albicans. Astaxanthin also protects human lymphocytes and neutrophils against the oxidant stresses imposed by the actions of certain white blood cells without reducing the killing effects of white blood cells themselves.
Epidemiological studies reveal that increased intake of carot-

enoids such as astaxanthin typically lowers risk of many different types of cancer. In this sense, the positive role of astaxanthin in the prevention of different types of cancer, such as colon and breast cancer has been demonstrated.
Finally, different works also support the effect of astaxanthin on protecting tissues from the impact of obesity and diabetes, shielding blood vessel cells from damage and slowing cognitive decline. Research reviews support the assumption that protecting body tissues from oxidative damage with daily ingestion of natural astaxanthin might be a practical and beneficial strategy in health management.

c. Canthaxanthin

Canthaxanthin administration effectively acted as a chemopreventer in chemically and/or physically induced cancers in rats. Cell lines responsive to the antitumour effects of canthaxanthin included murine melanoma, fibrosarcoma and human squamous carcinoma. Moreover, canthaxanthin decreased the extent of malignant transformation induced by both methylcholanthrene and by X-ray treatment.

Canthaxanthin has been reported to act as an antioxidant, to potentiate immune response, to enhance gap junctional communication between cells directly or through the formation of 4-oxo-retinoic acid, which is also able to stimulate the retinoic acid receptor. All these functions have been discussed as possible mechanisms involved in the anti-tumour effect of this compound.
It has been suggested that apoptosis may be inhibited in cancer cells and that several anti-tumour agents may activate it, producing morphological and biochemical events, such as cellular shrinkage, chromatin condensation, DNA breaks and fragmentation. Dietary and/or pharmacological manipulation of apoptosis may underlie novel treatment strategies to protect from cancer. In line with this, the possibility that canthaxanthin can reduce cell

growth by inducing apoptosis has been investigated, demonstrating that canthaxanthin is able to induce apoptosis in tumour cells.

d. Capsanthin

Ingestion of paprika extract obtained from red peppers, mainly rich in the carotenoid capsanthin, has shown to up-regulate different hepatic genes positively involved in the glucose metabolism and the expression of low-density lipoprotein receptors in rodents. On the other hand, a down-regulation of some genes involved in cholesterol catabolism has also been observed. This would result in a promotion of glucose and fatty acid metabolism and stabilisation of blood lipid level disorders. Capsanthin has also been shown to increase plasma HDL-cholesterol, which has been related to a lower incidence of cardivascular diseases.
Paprika extract has also been reported to be a cancer chemoprotective in in vitro and in vivo trials in rodents. Moreover, orally administered capsanthin has been suggested to specifically prevent colon carcinogenesis in humans. Additionally, a positive effect on chronic inflammation in adipocytes has also been shown. Most of these effects may be related to paprika extract's antioxidant properties. In fact, an improvement in endogenous antioxidant defences has been pointed out in humans that received smoked paprika in their diet.

8. REGULATORY ASPECTS IN THE EU

In the European Union, carotenoids used in animal nutrition are considered as feed additives and therefore are under the scope of the Feed Additives Regulation[4]. This regulation requires that feed additives such as carotenoids are subject to a pre-marketing authorisation.

8.1 Authorisation process

a. Assessment

As previously mentioned, carotenoids used in animal nutrition can only be placed on the market if they are already authorised or following the submission of a Technical dossier allowing their evaluation by the European Food Safety Authority (EFSA). The evaluation encompasses different aspects of the substance to be authorised. This covers the manufacturing process and the consequential specifications, the assessment of the information necessary to verify the safety of the product under its proposed conditions of use for the target species, for the consumer of products of animal origin, for the workers handling the products containing the substance and for the environment. Finally, the efficacy of the product for the relevant function (usually the pigmentation of the animal products) is also verified by the EFSA experts.

When all documents and information have been assessed by the EFSA experts, an opinion is published as the basis for the conditions of authorisation of the substance as a feed additive.

[4] For more information on this regulation, connect to the FEFANA website: www.fefana.org

b. Authorisation

When approved by vote by the Member States on proposal from the European Commission, a regulation authorizing the feed additive and establishing its specifications and conditions of use is published in the Official Journal of the European Union.

Authorised carotenoids are listed in the European Union Feed Additive Register[5] according to their main function in animal nutrition. The Feed Additive Register is a positive list and therefore only carotenoids that are listed and described in this Register can be used in animal nutrition. All carotenoids currently used in animal nutrition for colouring purpose are listed under the Category ‘Sensory Additives (2)’ and under the functional group ‘colourants (a)’, which includes the following subgroups: “Substances that add or restore colour in feedingstuffs (i)”; “substances which, when fed to animals, add colours to food of animal origin (ii)”; and “substances which favourably affect the colour of ornamental fish or birds (iii)”.

c. Conditions of use

The conditions of use of the carotenoids in feed are defined in the authorising regulation adopted by the Standing Committee of the Food Chain and Animal Health. For each of the carotenoids listed in the Feed Additive Register, such a regulation has been published and is accessible via a link within this Register. Carotenoids can therefore only be used in animal nutrition if they correspond to the description provided in the authorising regulation and when respecting the defined conditions of use.

The users of the carotenoids shall implement the relevant conditions of use stipulated in the authorising regulation. In particular, this can cover the way to incorporate the carotenoids in feed, such

[5] For more information on this regulation, connect to the FEFANA website: www.fefana.org

as direct inclusion or inclusion via a premixture. Finally, depending on the conditions set in the authorising regulation, carotenoids can be used in water for drinking. In this case, carotenoids cannot be distributed as such to farmers. Therefore, when promoting the use of carotenoids in water, the feed business operator shall prepare a suitable form of use, such as complementary feeds .

d. Holder of authorisation

In general, when registered as sensory additives, the carotenoids are authorised without a link to a specific authorisation holder. This means that any supplier of the substance can place his product on the market, as soon as the active substance meets the specification defined in the authorising regulation and if the other components of the formulation can be used in animal nutrition, i.e. comply with the different feed regulations.
When produced by fermentation using a Genetically Modified Microorganism or by extraction from a Genetically Modified Plant, the carotenoid can be authorised with a link to a specific holder. In this case, only the holder of the authorisation or authorised companies (through license) can place the additive on the market.
Due to their other biological functions, carotenoids could potentially be authorised as zootechnical additives. In this case, the specific form of the additive would be described and the authorisation would be linked to a specific authorisation holder, meaning that this application can only be claimed when the product from the authorised holder is used.

8.2 Other Regulatory considerations

a. Other sources of Carotenoids

Carotenoids are normally incorporated in the feed in the form of feed additives (see chapter IV). However, some milled flowers or plants are also used in animal nutrition. These plants containing high level of authorised carotenoids (e.g. tagetes or paprika) are considered as functional feed ingredients. However, bioavailability of carotenoids from those sources is very low compared to the plant extracts. In order to classify the product containing the carotenoids to be placed on the market or to be used, it is advisable to use the FEFANA classification tool .

b. Feed Hygiene Regulation

The production and use of carotenoids are covered, as for any other feed products, by the scope of the Feed Hygiene Regulation1. The Feed Hygiene Regulation defines the rules under which feed shall be produced and used in animal nutrition, with a view to guarantee a high level of food safety.
According to this regulation, the manufacture of carotenoids shall be conducted in a hygienic way, following the highest quality rules as well as the evaluation of the process according to the Hazard Analysis and Critical Control Point (HACCP) methodology. In addition, the regulation stipulates that the manufacture of carotenoids can only be run in production sites that have received an approval from the local control authorities. This means that for each new manufacturing site a pre-evaluation of the site is required by the authorities before production. This is based on an inspection from the control authorities, which give an approval number to the site. This approval number will be published officially in the production country and must be indicated on the labels by the operator.
Carotenoid products being produced in third countries can also

be marketed within the EU, whenever following the specifications and conditions of use set up on the authorizing regulation. In this case, the label must show the approval number of the European operator who first places the product within the EU market.
The users of carotenoids (premix manufacturers and feed millers) shall also follow the rules of hygienic production of their products and their sites.

9. REFERENCES

1. Natural occurrence and classification

- Armstrong G.A., Hearst J.E. 1996. Genetics and molecular biology of carotenoid pigment biosynthesis. The FASEB Journal, 10:228-237.
- Bartley G.E., Scolnik P.A. 1995. Plant carotenoids: pigments for photoprotection, visual attraction, and human health. The Plant Cell, 7:1027-1038.
- Breithaupt D.E. 2007. Modern application of xanthophylls in animal feeding – a review. Trends in Food Science & Technology, 18:501-506.
- Britton G. 1995. Structure and properties of carotenoids in relation to function. The FASEB Journal, 9:1551-1558.
- Farré G., Sanahuja G., Naqvi S., Bai C., Capell T., Zhu C., Christou P. 2010. Travel advice on the road to carotenoids in plants. Plant science, 179:28-48.
- Cazzonelli C. I. 2011. Carotenoids in nature: insights from plants and beyond. Functional Plant Biology, 38:833-847.
- Deming D.M., Erdmand J.W. Jr. 1999. Mammalian carotenoid absorption and metabolism. Pure and Applied Chemistry, 71:2213-2223.
- Johnson E.A., Schroeder W.A. 1995. Microbial carotenoids. In: Advances in Biochemical Engineering Biotechnology – Vol. 53. Ed.: Fiechter A. Springer, Berlin, pp. 119-178.
- Jin E., Polle J.E.W., Lee H.K., Hyun S.M., Chang M. 2003. Xanthophylls in microalgae: from biosynthesis to biothecnological mass production and application. Journal of Microbiology and Biotechnology, 13:165-174.
- Kopsell D.A., Kopsell D.E. 2006. Accumulation and bioavailability of dietary carotenoids in vegetable crops. Trends in Plant Science, 11:499-507.
- Maoka T. 2011. Carotenoids in Marine Animals. Marine Drugs, 9:278-293.

- Marusich W.L., Bauernfeind J.C. 1981. Oxycarotenoids in poultry feeds. In: Carotenoids as Colourants and Vitamin A Precursors. Ed.: Bauernfeind J.C. Academic Press, New York, pp. 319-462.
- Matsuno T. 2001. Aquatic animal carotenoids. Fisheries Science, 67:771-783.
- • Moran N.A., Jarvik T. 2010. Lateral transfer of genes from fungi underlies carotenoid production in aphids. Science, 328:624–627.
- Nelis H.J., De Leenheer A.P. 1991. Microbial sources of carotenoid pigments used in foods and feeds. Journal of Applied Bacteriology, 70:181-191.
- Ötles S., Çagindi Ö. 2008. Carotenoids as natural colourants. In: Food colourants: Chemical and Functional Properties. Ed. C. Socaciu, CRC Press. Boca Raton, F.L. pp. 51-70.
- Schiedt K. 1998. Absorption and metabolism of carotenoids in birds, fish and crustaceans. In: Carotenoids – Volume 3: Biosynthesis and Metabolism. Eds: Britton G., Liaaen-Jensen S., Pfander H. Birkhäuser Verlag, Basel, Switzerland. pp. 285-355.
- Slifka K.A., Bowen P.E., Stacewicz-Sapuntzakis M., Crissey S. D. 1999. A survey of serum and dietary carotenoids in captive wild animals. Journal of Nutrition, 129:380-390.

2. Role of carotenoids

- Bédécarrats G.Y., Leeson S. 2006. Dietary lutein influences immune response in laying hens. Journal of Applied Poultry Research, 15:183–189.
- Brown L., Rimm E.B., Seddon J.M., Giovannucci E.L., Chasan-Taber L., Spiegelman D., Willett W.C., Hankinson S.E. 1999. A prospective study of carotenoid intake and risk of cataract extraction in US men. American Journal of Clinical Nutrition, 70:517-524.
- Cazzonelli C. 2011. Carotenoids in nature: insights from plants and beyond. Functional Plant Biology, 38: 833-847.
- Chien, Y.H., Jeng, S.C. 1992. Pigmentation of kuruma prawn, Penaeus japonicus bate, by various pigment sources and levels and feeding regimes. Aquaculture, 102:333-346.
- Christiansen R., Lie Ø., Torrissen O.J. 1994. Effect of astaxanthin and vitamin A on growth and survival during first feeding of Atlantic salmon, Salmo salar L. Aquaculture and Fish Management, 25:903-914.
- Christiansen R., Lie Ø., Torrissen O.J. 1995. Growth and survival of Atlantic salmon, Salmo salar L., fed different dietary levels of astaxanthin. First-feeding fry. Aquaculture Nutrition, 1:189-198.
- Czeczuga, B. 1979. Carotenoids in fish. XIX. Carotenoids in the eggs of Oncorhynchus keta (Walbaum). Hydrologia, 63:45-47.
- Fletcher D.L., Halloran R.H. 1983. Egg yolk pigmenting properties of a marigold extract and paprika oleoresin. Poultry Science, 62:1205-1210.
- Gao Y.Y., Xie Q.M, Ma J.Y., Zhang X.B., Zhu J.M., Shu D.M., Sun B.L., Jin L., Bi Y.Z. 2013. Supplementation of xanthophylls increased antioxidant capacity and decreased lipid peroxidation in hens and chicks. British Journal of Nutrition, 109:977-983.
- Gao Y.Y., Xie Q.M, Jin L., Sun B.L., Ji. J., Cheng F., Ma J.Y.,

Bi Y.Z. 2012. Supplementation of xanthophylls decreased pro-inflamatory and increased anti-inflammatory cytokines in hens and chicks. British Journal of Nutrition, 108:1746-1755.
- Kim H.W., Chew B.P., Wong T.S., Park J.S., Weng B.B., Byrne K.M., Hayek M.G., Reinhart G.A. 2002. Dietary lutein stimulates immune response in the canine. Veterinary Immunology and Immunopathology, 74:315-327.
- Kim H.W., Chew B.P., Wong T.S., Park J.S., Weng B.B., Byrne K.M., Hayek M.G., Reinhart G.A 2000. Modulation of humoral and cell-mediated immune responses by dietary lutein in cats. Veterinary Immunology and Immunopathology, 73:331-41.
- Kläui H., Bauernfeind J.C. 1981. Carotenoids as food colors. In: Carotenoids as Colorants and Vitamin A Precursors. Ed.: Bauernfeind J.C. Academic Press, New York, pp. 80.
- Koutsos, E.A. 2002. Carotenoids in avian immune systems. PhD thesis. University of California, Davis.
- Koutsos E.A., Cifford A.J., Calvert C.C., Klasing K.C. 2003. Maternal carotenoid status modifies the incorporation of dietary carotenoids into immune tissues of growing chickens (Gallus gallus domesticus). Journal of Nutrition, 133: 1132–1138.
- Koutsos E.A., García López J.C., Klasing K.C. 2006. Carotenoids from in ovo or dietary sources blunt systemic indices of the inflammatory response in growing chicks (Gallus gallus domesticus) Journal of Nutrition, 136: 1027–1031.
- Koutsos E.A., García López J.C., Klasing K.C. 2007. Maternal and dietary carotenoids interactively affect cutaneous basophil responses in growing chickens. Comparative Biochemistry and Physiology, 147:87-92.
- McGraw K.J., Beebee M.D., Hill G.E., Parker R.S. 2003. Lutein-based plumage coloration in songbirds is a consequence of selective pigment incorporation into feathers. Comparative Biochemistry and Physiology B, 135:689-696.
- McGraw K.J., Adkins-Regan E., Parker R.S. 2005. Maternally derived carotenoid pigments affect offspring survival, sex ratio, and sexual attractiveness in a colourful songbird. Naturwissenschaften, 92:375-380.
- Meriwether L.S., Humphrey B.D., Peterson D.G., Klasing K.C., Koutsos E.A. 2010. Lutein exposure, in ovo or in the diet, reduces parameters of inflammation in the liver and spleen laying-type chicks (Gallus gallus domesticus). Journal of Animal Physiology and Animal Nutrition, 94:115-122.
- Marusich W.L., Bauernfeind J.C. 1981. Oxycarotenoids in poultry feeds. In: Carotenoids as Colourants and Vitamin A Precursors. Ed.: Bauernfeind J.C. Academic Press, New York, pp. 319-462.
- Narisawa, T., Fukaura Y., Hasebe M., Ito M., Aizawa R., Murakoshi M., Uemura S., Khachik F., Nishino H. 1996. Inhibitory effects of natural carotenoids, α-carotene, β-carotene, lycopene and lutein on colonic aberrant crypt foci formation in rats. Cancer Letters, 107:137-142.
- Olson J.A. 1999a. Carotenoids. In: Modern nutrition in health and disease, 9th edition. Eds: Shils M.E., Olson J.A., Shike M., Ross A.C. Williams & Wilkins, Baltimore, pp 525-541.
- Olson J.A. 1999b. Carotenoids and human health. Archivos Latinoamericanos de Nutrición, 49:7-11.
- Olson J.A, Krinsky N.I. 1995. Introduction: the colourful, fascinating world of the carotenoids: important physiologic modulators. The FASEB Journal, 9:1547-50.
- Polívka T., Frank H.A. 2010.Molecular factors controlling photosynthetic light harvesting by carotenoids. Accounts of Chemical Research, 43:1125–1134.
- Renzi L.M., Johnson E.J. 2007. Lutein and age-related ocular disorders in the older adult: a Review. Journal of Nutrition for the Elderly, 26:139-157.
- Rosa A.P., Scher A., Sorbara J.O.B., Boemo L.S., Forgiarini J., Londero A. 2012. Effects of Canthaxanthin on the Productive and Reproductive Performance of Broiler Breeders. Poultry Science, 91:660–666.
- Selvaraj R.K., Koutsos E.A., Calvert C.C., Klasing K.C. 2006.

Dietary lutein and fat interact to modify macrophage properties in chicks hatched from carotenoid deplete or replete eggs. Journal of Animal Physiology and Animal Nutrition, 90:70-80
- Selvaraj R.K., Koutsos E.A., García-López J.C., Klasing K.C. 2007. Maternal and dietary carotenoids interactively affect cutaneous basophil responses in growing chickens (Gallus gallus domesticus). Comparative Biochemistry and Physiology B, 147:87-92.
- Surai P.F., Noble R.C., Speake B.K. 1996. Tissue-specific differences in antioxidant distribution and susceptibility to lipid peroxidation during development of the chick embryo. Biochimica et Biophysica Acta, 1304:1-10.
- Surai P.F. 2002. Natural antioxidants in avian nutrition and reproduction. Nottingham University Press, UK.
- Surai P.F. 2012a. The antioxidant properties of Canthaxanthin and its potential effects in the poultry eggs and on embryonic development of the chick. Part 1. World's Poultry Science Journal, 68:465-475.
- Surai P.F. 2012b. The antioxidant properties of Canthaxanthin and its potential effects in the poultry eggs and on embryonic development of the chick. Part 2. World's Poultry Science Journal, 68:717-726.
- Wald N.J., Thompson S.G., Densem J.W., Boreham J., Bailey A. 1988. Serum β-carotene and subsequent risk of cancer: results from the BUPA study. British Journal of Cancer, 57:428-433.
- Yamada S., Tanaka Y., Sameshima M., Ito Y. 1990. Pigmentation of prawn (Penaeus japonicus) with carotenoids: I. Effect of dietary astaxanthin, β-carotene and canthaxanthin on pigmentation. Aquaculture, 87: 323-330.

3. Processing of carotenoids as feed additives

- Breithaupt D.E. 2007. Modern application of xanthophylls in animal feeding – a review. Trends in Food Science & Technology, 18:501-506.
- Britton G. 1995. Structure and properties of carotenoids in relation to function. The FASEB Journal, 9:1551-1558.
- Johnson E.A., Schroeder W.A. 1995. Microbial carotenoids. In: Advances in Biochemical Engineering Biotechnology – Vol. 53. Ed.: Fiechter A. Springer, Berlin, pp. 119-178.
- Jin E., Polle J.E.W., Lee H.K., Hyun S.M., Chang M. 2003. Xanthophylls in microalgae: from biosynthesis to biothecnological mass production and application. Journal of Microbiology and Biotechnology, 13:165-174.
- Kläui H., Bauernfeind J.C. 1981. Carotenoids as food colors. In: Carotenoids as Colorants and Vitamin A Precursors. Ed.: Bauernfeind J.V. Academic Press, New York, pp. 80.
- Kopsell D. A., Kopsell D. E. 2006. Accumulation and bioavailability of dietary carotenoids in vegetable crops. Trends in Plant Science, 11:499-507.
- Marusich W.L., Bauernfeind J.C. 1981. Oxycarotenoids in poultry feeds. In: Carotenoids as Colourants and Vitamin A Precursors. Ed.: Bauernfeind J.C. Academic Press, New York, pp. 319-462.
- Nelis H.J., De Leenheer A.P. 1991. Microbial sources of carotenoid pigments used in foods and feeds. Journal of Applied Bacteriology, 70:181-191.
- Nozière P., Graulet B., Lucas A., Martin B. 2006. Carotenoids for ruminants: from forages to dairy products. Animal Feed Science and Technology, 131:418-450.
- Pfanfer H., Liaaen-Jensen S., Britton G. 1996. General Aspects - Synthesis in perspective. In: Carotenoids – Vol. 2: Synthesis. Eds: Britton G., Liaaen-Jensen S., Pfander H. Birkhäuser Verlag, Basel, Switzerland, pp. 1-6.
- Schiedt K. 1998. Absorption and metabolism of carotenoids

in birds, fish and crustaceans. In: Carotenoids – Vol. 3: Biosynthesis and Metabolism. Eds Britton G., Liaaen-Jensen S., Pfander H., Birkhäuser Verlag, Basel, Switzerland. pp. 285-355.
- Soukup M., Spurr P., Widmer E. 1996. General Aspects - Strategies for building the carbon skeleton. In: Carotenoids – Vol. 2: Synthesis. Eds: Britton G., Liaaen-Jensen S., Pfander H. Birkhäuser Verlag, Basel, Switzerland, pp. 7-14.

4. Conditions of use of carotenoids

- Bédécarrats G.Y., Leeson S. 2006. Dietary lutein influences immune response in laying hens. Journal of Applied Poultry Research, 15:183–189.
- Bjerkeng B., Storebakken T., Liaaen-Jensen S. 1990. Dose response to carotenoids by rainbow trout in the sea: resorption and metabolism of dietary astaxanthin and canthaxanthin. Aquaculture, 91:153-162.
- Kim H.W., Chew B.P., Wong T.S., Park J.S., Weng B.B., Byrne K.M., Hayek M.G., Reinhart G.A. 2000. Modulation of humoral and cell-mediated immune responses by dietary lutein in cats. Veterinary Immunology and Immunopathology, 73:331-341.
- Kim H.W., Chew B.P., Wong T.S., Park J.S., Weng B.B., Byrne K.M., Hayek M.G., Reinhart G.A. 2000. Dietary lutein stimulates immune response in the canine. Veterinary Immunology and Immunopathology, 74:315-327.
- Koutsos E.A., García López J.C., Klasing K.C. 2007. Maternal and dietary carotenoids interactively affect cutaneous basophil responses in growing chickens (Gallus gallus domesticus). Comparative Biochemistry and Physiology B, 147:187-192.
- Marusich W. L., Bauernfeind J. C. 1981. Oxycarotenoids in poultry feeds. In: Carotenoids as Colourants and Vitamin A Precursors. Ed.: Bauernfeind J.C. Academic Press, New York, pp. 319-462.

- Selvaraj R.K., Koutsos E.A., Calvert C.C., Klasing K.C., 2006. Dietary lutein and fat interact to modify macrophage properties in chicks hatched from carotenoid deplete or replete eggs. Journal of Animal Physiology and Animal Nutrition, 90:70-80
- Torrissen O.J., Christiansen R., Struksnæs G., Estermann R. 1995. Astaxanthin deposition in the flesh of Atlantic salmon, Salmo salar L., in relation to dietary astaxanthin concentration and feeding period. Aquaculture Nutrition, 1:77-84.

7. Benefits for consumers and food products

- Aizawa K., Matsumoto T., Inakuma T., Ishijima T., Nakai Y., Abe K., Amano F. 2009. Administration of tomato and paprika beverages modifies hepatic glucose and lipid metabolism in mice: a DNA microarray analysis. Journal of Agricultural and Food Chemistry, 57:10964-10971.
- Alves-Rodrigues A., Shao A. 2004. The science behind lutein. Toxicology Letters, 150:57-83.
- Bendich A., Shapiro S.S. 1986. Effect of b-carotene and canthaxanthin on the immune responses of the rat. Journal of Nutrition, 116:2254–2262.
- Bolin A.P., Guerra B.A., Nascimento S.J., Otton R. 2012. Changes in lymphocyte oxidant/antioxidant parameters after carbonyl and antioxidant exposure. International Immunopharmacology, 14:690-697.
- Bone R. A., Landrum J. T., Dixon Z., Chen Y., Llenera C. 2000. Lutein and zeaxanthin in the eyes, serum and diet of human subjects. Experimental Eye Research, 71:239-245.
- Camera E., Mastrofrancesco A., Fabbri C., Daubrawa F., Picardo M., Sies H., Stahl W. 2009. Astaxanthin, canthaxanthin and beta-carotene differently affect UVA-induced oxidative damage and expression of oxidative stress- responsive enzymes. Experimental Dermatology, 18:222-231.
- Choi H.D., Kim J.H., Chang M.J., Kyu-Youn Y., Shin W.G. 2011. Effects of astaxanthin on oxidative stress in overweight

and obese adults. Phytotherapy Research, 25:1813-1818.
- Chung H.-Y., Rasmussen H. M., Johnson E. J. 2004. Lutein bioavailability is higher from lutein-enriched eggs than from supplements and spinach in men. The Journal of Nutrition, 134:1887-1893.
- Grubbs C.J., Eto I., Juliana M.M., Whitaker L.M. 1991. Effect of canthaxanthin on chemically induced mammary carcinogenesis. Oncology, 48:239–245.
- Guerin M., Huntley M.E., Olaizola M. 2003. Haematococcus astaxanthin: applications for human health and nutrition. Trends in Biotechnology, 21:210-216.
- Hanusch M., Stahl W., Schulz W.A., Sies H. 1995. Induction of gap junctional communication by 4-oxoretinoic acid generated from its precursor canthaxanthin. Archives of Biochemistry and Biophysics, 317:423–428.
- Huang D.S., Odeleye O.E., Watson R.R. 1992. Inhibitory effects of canthaxanthin on in vitro growth of murine tumour cells. Cancer Letters, 65:209–213.
- Hughes D. A. 2001. Dietary carotenoids and human immune function. Nutrition, 10:823-827.
- Ikeuchi M., Koyama T., Takahashi J., Yazawa K. 2007. Effects of astaxanthin in obese mice fed a high-fat diet. Bioscience, Biotechnology and Biochemstry, 71:893-899.
- Katagiri M., Satoh A., Tsuji S., Shirasawa T. 2012. Effects of astaxanthin-rich Haematococcus pluvialis extract on cognitive function: a randomised, double-blind, placebo-controlled study. Journal of Clinical Biochemistry and Nutrition, 51:102-107.
- Katsumura N., Okuno M., Onogi N., Moriwaki H., Muto Y., Kojima S. 1996. Suppression of mouse skin papilloma by canthaxanthin and b-carotene in vivo: possibility of the regression of tumorigenesis by carotenoids without conversion to retinoic acid. Nutrition and Cancer, 26:203–208.
- Kennedy A.R., Krinsky N.I. 1994. Effects of retinoids, b-carotene and canthaxanthin on UV- and X-ray-induced transformation of C3H10T1/2 cells in vitro. Nutrition and Cancer, 22:219–232.

- Kerr J.F.R., Harmon B.V. 1991. Definition and incidence of apoptosis: an historical perspective. In: Apoptosis: the Molecular Basis of Cell Death. Eds: Tomei L.D., Cope F.O. Cold Spring Harbor Laboratory Press, New York, pp. 5–29.
- Kim S., Ha T., Hwang I.K. 2009. Analysis, bioavailability, and potential healthy effects of capsanthin, natural red pigment from Capsicum spp. Food Reviews International, 25:198–213.
- Lee S.J., Bai S.K., Lee K.S., Namkoong S., Na H.J., Ha K.S., Han J.A., Yim S.V., Chang K., Kwon Y.G., Lee S.K., Kim, Y.M. 2003. Astaxanthin inhibits nitric oxide production and inflammatory gene expression by suppressing I(kappa)B kinase-dependent NF-kappaB activation. Molecules and Cells, 16:97-105.
- Lu Y.P., Liu S.Y., Sun H., Wu X.M., Li J.J., Zhu L. 2010. Neuroprotective effect of astaxanthin on H(2)O(2)-induced neurotoxicity in vitro and on focal cerebral ischemia in vivo. Brain Research, 1360:40-48.
- Macedo R.C., Bolin A.P., Marin D.P., Otton R. 2010. Astaxanthin addition improves human neutrophils function: in vitro study. European Journal of Nutrition, 49:447-457.
- Maeda H., Saito S., Nakamura N., Maoka T. 2013. Paprika pigments attenuate obesity-induced inflammation in 3T3-L1 adipocytes. ISRN Inflammation, 2013:article ID 763758, 9p.
- Maoka T., Mochida K., Kozuka M., Ito Y., Fujiwara Y., Hashimoto K., Enjo F., Ogata M., Nobukuni Y., Tokuda H. 2001. Cancer chemopreventive activity of carotenoids in the fruits of red paprika (Capsicum annuum L.). Cancer Letters, 172:103-109.
- Nagaki Y., Mihara M., Takahashi J., Kitamura A., Horita Y., Sugiura Y., Tsukahara H. 2005. The effect of astaxanthin on retinal capillary blood flow in normal volunteers. Journal of Clinical Therapeutics & Medicines, 21:537-542.
- Nagendraprabhu P., Sudhandiran G. 2011. Astaxanthin inhib-

its tumor invasion by decreasing extracellular matrix production and induces apoptosis in experimental rat colon carcinogenesis by modulating the expressions of ERK-2, NFkB and COX-2. Investigational New Drugs, 29:207-224.
- Naito Y., Uchiyama K., Aoi W., Hasegawa G., Nakamura N., Yoshida N., Maoka T., Takahashi J., Yoshikawa, T. 2004. Prevention of diabetic nephropathy by treatment with astaxanthin in diabetic db/db mice. Biofactors, 20:49-59.
- Nakamura A., Isobe R., Otaka Y., Abematsu Y., Nakata D., Honma C., Sakurai S., Shimada Y., Horiguchi M. 2004. Changes in visual function following peroral astaxanthin. Japanese Journal of Clinical Ophthalmology, 58:1051-1054.
- Nakao R., Nelson O.L., Park J.S., Mathison B.D., Thompson P.A., Chew B.P. 2010. Effect of dietary astaxanthin at different stages of mammary tumor initiation in BALB/c mice. Anticancer Research, 30:2171-2175.
- Narisawa T., Fukaura Y., Hasebe M., Nomura S., Oshima S., Inakuma T. 2000. Prevention of N-Methylnitrosourea-induced colon carcinogenesis in rats by oxygenated carotenoid capsanthin and capsanthin-rich paprika juice. Proceedings of the Society for Experimental Biology and Medicine, 224:116-122.
- Nikawa T., Schulz W.A., van den Brink C.E., Hanusch M., van der Saag P., Stahl W., Sies H. 1995. Efficacy of all-trans-b-carotene, canthaxanthin, and all-trans-9-cis-, and 4-oxoretinoic acids in inducing differentiation of an F9 embryonal carcinoma RAR-lacZ reporter cell line. Archives of Biochemistry and Biophysics, 316:65–672.
- Ohmori T., Podack E.R., Nishio K., Takahashi M., Miyahara Y., Takeda Y., Kubota N., Funayama Y., Ogasawara H., Ohira T., Ohta S., Saijo N. 1993. Apoptosis of lung cancer cells caused by some anti-cancer agents (MMC, CPT-11, ADM) is inhibited by bcl-2. Biochemical and Biophysical Research Communications, 192:30–36.
- Palozza P., Luberto C., Ricci P., Sgarlata E., Calviello G., Bartoli G.M. 1996. Effect of b-carotene and canthaxanthin on tert-butyl hydroperoxideinduced lipid peroxidation in murine normal and tumour thymocytes. Archives of Biochemistry and Biophysics, 325:145–151.

- Palozza P., Maggiano N., Calviello G., Lanza P., Piccioni E., Ranelletti F.O., Bartoli G.M. 1998. Canthaxanthin induces apoptosis in human cancer cell lines. Carcinogenesis 19:373-376
- Palozza P., Torelli C., Boninsegna, A., Simone R., Catalano A., Mele M.C., Picci N. 2009. Growth-inhibitory effects of the astaxanthin-rich alga Haematococcus pluvialis in human colon cancer cells. Cancer Letters, 283:108-117.
- Palozza P.,Krinsky N.I. 1992. Astaxanthin and canthaxanthin are potent antioxidants in a membrane model. Archives of Biochemistry and Biophysics, 297:291–295.
- Pung A., Rundhaug J.E., Yoshizawa C.N., Bertram J.S. 1988. b-Carotene and canthaxanthin inhibit chemically- and physically-induced neoplastic transformation in 10T1/2 cells. Carcinogenesis, 9:1533–1539.
- Raff M.C. 1992. Social controls on cell survival and cell death. Nature, 356:397–400.
- Sasaki Y., Kobara N., Higashino S., Giddings J.C., Yamamoto J. 2011. Astaxanthin inhibits thrombosis in cerebral vessels of stroke-prone spontaneously hypertensive rats. Nutrition Research, 31:784-789.
- Suganuma K., Nakajima H., Ohtsuki M., Imokawa G. 2010. Astaxanthin attenuates the UVA-induced up-regulation of matrix-metalloproteinase-1 and skin fibroblast elastase in human dermal fibroblasts. Journal of Dermatological Science, 58:136-142.
- Tanaka T., Kawamori T., Ohnishi M., Makita H., Mori H., Satoh K., Hara A. 1995. Suppression of azoxymethane-induced rat colon carcinogenesis by dietary administration of naturally occurring xanthophylls astaxanthin and canthaxanthin during the postinitiation phase. Carcinogenesis, 16:2957–2963.
- Tanaka T., Makita H., Ohnishi M., Mori H., Satoh K., Hara A.

1995. Chemoprevention of rat oral carcinogenesis by naturally occurring xanthophylls asthaxanthin and canthaxanthin. Cancer Research, 55:4059–4064.

- Tormo M.A., Campillo J.E., Viña J., Gómez-Encinas J., Borrás C., Torres M.D., Campillo C. 2013. The mechanism of the antioxidant effect of smoked paprika from La Vera, Spain. Journal of Food, 11:114-118.
- Zhang L.X., Cooney K.W., Bertram J.S. 1992. Carotenoids upregulate connexin 43 gene expression independent of their provitamin A or antioxidant properties. Cancer Research, 52:5707–5712.

Acknowledgements

This booklet is the result of efforts and commitment of the members of the FEFANA Working Group Carotenoids and in particular of the following companies: DSM, ITPSA and NOVUS. Acknowledgements for their commitment in writing this text go to: Elkin AMAYA, Philippe BECQUET, Sergi CARNÉ, Silvia PERIS and Pilar MIRALLES.

For the pictures courtesy of :
- Figure 6: Farré et al. 2010 Plant Science 179: 28-48.
- Figure 9: A. Borràs and J. C. Senar (Natural History Museum of Barcelona)
- Figure 10: Elkin Amaya.
- Figure 14: Kläui H., Bauernfeind J. C. 1981. Carotenoids as food colors. In: Carotenoids as Colorants and Vitamin A Precursors. Ed. J. C. Bauernfeind., Academic Press, New York, pp. 80.

And also:
- ITPSA for figures 3, 4 and 5.
- DSM for the cover, and for figures 2, 7, 8, 11, 12, 13, 15, 16, 17, 18, 19 and 20.

Disclaimer and Copyright

This booklet is intended to provide the best-of-our-knowledge basic information to anyone interested to get a better understanding about Carotenoids in Animal Nutrition. However, FEFANA does not take any responsibility for whatever use of the information provided herewith, by either the general public or any actor in the food and feed chain. For more detailed information on specific use please refer to the technical documentation and safety data sheets as provided by supplier.

www.fefana.org

ISBN 978-2-9601289-4-9

fefana

Avenue Louise, 130A Box 1 - 1050 Brussels - Belgium
Tel.: +32 (0)2 639.66.60 - Fax: +32 (0)2 640.41.11
E-mail: info@fefana.org