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Chapter 2

Microalgae in Novel Food Products

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Abstract

The implications of diet on health sustainability have assumed a major importance, supported by considerable epidemiological evidences, and is well recognized by the scientific community and general public, on developed countries. Microalgae are able to enhance the nutritional content of conventional food and feed preparation and hence to positively affect humans and animal health due to their original chemical composition, namely high protein content, with balanced amino acids pattern, carotenoids, fatty acids, vitamins, polysaccharides, sterols, phycobilins and other biologically active compounds, more efficiently than traditional crops. The aim of this chapter is to review the most important features of microalgae in animal and human nutrition, particularly in the development of novel design-foods rich in carotenoids and polyunsaturated fatty acids with antioxidant effect and other beneficial health properties.

1. Introduction

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Modern food industry leads to an increase of cheaper, healthier and more convenient products. The use of natural ingredients, like polyunsaturated fatty acids (PUFA's) and antioxidant pigments, exhibiting high impact on functional properties is important to reduce chronic diseases incidence, which are strongly considered of capital importance in Europe, where aging population and welfare costs are fatal for public resources management. The impact of natural substances introduced in the diet via "usual" foods is proved to be efficient at long term and do not present the drawbacks of traditional therapeutic actions based on medicines of short term impact.

Microalgae are an enormous biological resource, representing one of the most promising sources for new products and applications (Pulz and Gross, 2004). They can be used to enhance the nutritional value of food and animal feed, due to their well balanced chemical composition. Moreover, they are cultivated as a source of highly valuable molecules such as polyunsaturated fatty acids, pigments, antioxidants, pharmaceuticals and other biologically active compounds. The application of microalgal biomass and/or metabolites is an interesting and innovative approach for the development of healthier food products.

Microalgal biotechnology is similar to conventional agriculture, but has received quite a lot of attention over the last decades, because they can reach substantially higher productivities than traditional crops and can be extended into areas and climates unsuitable for agricultural purposes (e.g. desert and seashore lands). Microalgae production is an important natural mechanism to reduce the excess of atmospheric CO_2 by biofixation and recycling of fixed C in products, ensuring a lower greenhouse effect, reducing the global environmental heating and climate changes. Microalgae cultivation also presents less or no seasonality, are important as feed to aquaculture and life-support systems, and can effectively remove nutrients (or pollutants) (e.g nitrogen and phosphorus) from water. Microalgal systems for sunlight driven environmental and production applications can clearly contribute to sustainable development and improved management of natural resources. Lately, microalgae have been seen with a great potential as a sustainable feedstock for biodiesel production, in substitution for oil from vegetable crops (Campbell, 1997), and also for hydrogen production (Dutta *et al*., 2005).

This chapter reviews the main applications of microalgae in feed and food products focusing the authors' work on this subject, for the last years.

2. Microalgae

Microalgae use by indigenous populations has occurred for centuries. However, the cultivation of microalgae is only a few decades old (Borowitzka, 1999) and among the 30000 species that are believed to exist (Chaumont, 1993, Radmer and Parker, 1994), only a few thousands strains are kept in collections, a few hundred are investigated for chemical content and just a handful are cultivated in industrial quantities (Olaizola, 2003).

Some of the most biotechnologically relevant microalgae are the green algae (Chlorophycea) *Chlorella vulgaris*, *Haematococcus pluvialis*, *Dunaliella salina* and the Cyanobacteria *Spirulina maxima* which are already widely commercialized and used, mainly as nutritional supplements for humans and as animal feed additives.

Chlorella vulgaris has been used as an alternative medicine in the Far East since ancient times and it is known as a traditional food in the Orient. It is widely produced and marketed as a food supplement in many countries, including China, Japan, Europe and the US, despite not possessing GRAS status. *Chlorella* is being considered as a potential source of a wide spectrum of nutrients (e.g. carotenoids, vitamins, minerals) being widely used in the healthy food market as well as for animal feed and aquaculture. *Chlorella* is important as a health promoting factor on many kinds of disorders such as gastric ulcers, wounds, constipation, anemia, hypertension, diabetes infant malnutrition and neurosis (Yamaguchi, 1997). It is also attributed a preventive action against atherosclerosis and hypercholesterolemia by glycolipids and phospholipids, and antitumor actions by glicoproteins, peptides and nucleotides (Yamaguchi, 1997). However the most important substance in *Chlorella* seems to be a beta-1,3-glucan, which is an active immunostimulator, a free-radical scavenger and a reducer of blood lipids (Spolaore *et al*., 2006).

Haematococcus pluvialis has been identified as the organism which can accumulate the highest level of astaxanthin in nature (1.5-3.0% dry weight). This carotenoid pigment is a potent radical scavenger and singlet oxygen quencher, with increasing amount of evidence suggesting that surpasses the antioxidant benefits of β-carotene, vitamin C and vitamin E. *Haematococcus* is currently the prime natural source of this pigment for commercial exploitation, particularly in aquaculture salmon and trout farming (Lorenz and Cysewski, 2000). Another natural source, *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) yeast requires a large amount of feed for sufficient pigmentation (Dufossé *et al*., 2005).

Dunaliella salina is an halotolerant microalga, naturally occurring in salted lakes, that is able to accumulate very large amounts of β-carotene, a valuable chemical mainly used as natural food colouring and provitamin A (retinol). The *D. salina* community in Pink Lake, Victoria (Australia) was estimated to contain up to 14% of this carotenoid in their dry weight (Aasen *et al*., 1969), and in culture some *Dunaliella* strains may also contain up to 10% and more β-carotene, under nutrient-stressed, high salt and high light conditions (Ben-Amotz and Avron, 1980; Oren, 2005). Apart from β-carotene *Dunaliella* produces another valuable chemical, glycerol.

Arthrosphira (*Spirulina*) grows profusely in certain alkaline lakes in Mexico and Africa and has been used as food by local populations since ancient times (Yamaguchi, 1997). It is extensively produced around the world (3000 tons/year) and broadly used in food and feed supplements, due of its high protein content and its excellent nutritive value, such as high γ-linolenic acid level (Ötles and Pire, 2001, Shimamatsu, 2004). In addition, this microalga has various possible health promoting effects: the alleviation of hyperlipidemia, suppression of hypertension, protection against renal failure, growth promotion of intestinal *Lactobacillus*, suppression of elevated serum glucose level (Spolaore *et al*., 2006), anticarcinogenic effect and have hypocholesterolemic properties (Reinehr and Costa, 2006). *Spirulina* is also the main source of natural phycocyanin, used as a natural food and cosmetic colouring (blue colour extract) and as biochemical tracer in immunoassays, among other uses (Ötles and Pire, 2001, Kato 1994, Shimamatsu, 2004).

Recently, attention has been drawn on the marine microalgae *Isochrysis galbana* and *Diacronema vlkianum* (Haptophyceae) due to their ability to produce long chain polyunsaturated fatty acids (LC-PUFA), mainly eicosapentaenoic acid (EPA, 20:5ω3) and

also docosahexaenoic acid (DHA, 22:6ω3), that are accumulated as oil droplets in prominent lipid bodies in the cell (Liu and Lin, 2001). These microalgae have been used as a feed species for commercial rearing of many aquatic animals, particularly larval and juvenile molluscs, crustacean and fish species (Fidalgo *et al*., 1998). For example, in a relative ranking of microalgal diets for clam *Mercenaria mercenaria*, the microalga *I. galbana* was shown as the most suitable source of nutritional for rapid growth (Wikfors *et al*., 1992), while D. *vlkianum* resulted in high growth rates and low mortality for the Pacific oyster *Crassostrea gigas* larvae (Ponis *et al*., 2006). These microalgae are also potentially promising for the food industry as a valuable source of LC-PUFA's, in alternative to fish oils, supplying also sterols, tocopherols, colouring pigments and other nutraceuticals (Bandarra *et al*., 2003; Donato *et al*., 2003).

3. Bioactive Molecules

As with any higher plant, the chemical composition of algae is not an intrinsic constant factor but varies over a wide range. Environmental factors, such as temperature, illumination, pH-value, mineral contents, CO_2 supply, or population density, growth phase and algae physiology, can greatly modified chemical composition. Table 1 presents indicative values of a gross chemical composition of different algae and compared with the composition of selected conventional foodstuffs.

Microalgae can biosynthesize, metabolize, accumulate and secrete a great diversity of primary and secondary metabolites, many of which are valuable substances with potential applications in the food, pharmaceutical and cosmetics industries (Yamaguchi, 1997).

3.1. Pigments

One of the most obvious and arresting characteristic of the algae is their colour. In general, each phylum has its own particular combination of pigments and an individual colour. Aside chlorophylls, as the primary photosynthetic pigment, microalgae also form various accessory or secondary pigments, such as phycobiliproteins and a wide range of carotenoids. These natural pigments are able to improve the efficiency of light energy utilization of the algae and protect them against solar radiation and related effects. Their function as antioxidants in the plant shows interesting parallels with their potential role as antioxidants in foods and humans (Van den Berg *et al*., 2000). Therefore, microalgae are recognized as an excellent source of natural colorants and nutraceuticals and it is expected they will surpass synthetics as well as other natural sources due to their sustainability of production and renewable nature (Dufossé *et al*., 2005).

Table 1. General composition of different human sources and microalgae (% dry matter) (adapted from Becker, 1994, Spolaore *et al*., 2006 and Natrah *et al*., 2007)

Commodity	Protein	Carbohydrate	Lipid
Baker's yeast	39	38	1
Meat	43	1	34
Egg	47	4	41
Milk	26	38	28
Rice	8	77	2
Soya	37	30	20
Anabaena cylindrical	43-56	25-30	4-7
Chaetoceros calcitrans	36	27	15
Chlamydomonas rheinhardii	48	17	21
Chlorella pyrenoidosa	57	26	2
Chlorella vulgaris	51-58	12-17	14-22
*Chlorella vulgaris**	38	33	5
Chlorella vulgaris	12	25	28
(carotenogenic)*	38	25	18
*Diacronema vlkianum**	57	32	6
Dunaliella salina	39-61	14-18	14-20
Euglena gracilis	10	40	41
Haematococcus pluvialis	48	27	15
(carotenogenic)*	28-39	40-57	9-14
Isochrysis galbana	50-56	10-17	12-14
Porphyridium cruentum	8-18	21-52	16-40
Scenedesmus obiquus	6-20	33-64	11-21
Scenedesmus dimorphus	60-71	13-16	6-7
Spirogyra sp.	45	21	4
Spirulina maxima	46-63	8-14	4-9
*Spirulina maxima**	63	15	11
Spirulina platensis	52	15	3
Synechococcus sp.			
Tetraselmis maculate			

*Values experimentally determined by the authors (Batista *et al*., 2007a).

3.1.1. Chlorophylls

All algae contain one or more type of chlorophyll: chlorophyll-*a* is the primary photosynthetic pigment in all algae (Figure 1) and is the only chlorophyll in cyanobacteria (blue-green algae) and rhodophyta. Like all higher plants, chlorophyta and euglenophyta contain chlorophyll-*b* as well; chlorophylls -*c*, -*d* and –*e* can be found in several marine algae and fresh-water diatoms. Chlorophylls amounts are usually about 0.5-1.5% of dry weight (Becker, 1994).

Apart from their use as food and pharmaceutical colorants, chlorophyll derivatives can exhibit health promoting activities. These compounds have been traditionally used in medicine due to its wound healing and anti-inflammatory properties as well as control of calcium oxalate crystals and internal deodorization (Ferruzi and Blakeslee, 2007). Recent epidemiological studies from *The Netherlands Cohort Study* (Balder *et al*., 2006) has provided evidence linking chlorophyll consumption to a decreased risk of colorectal cancer.

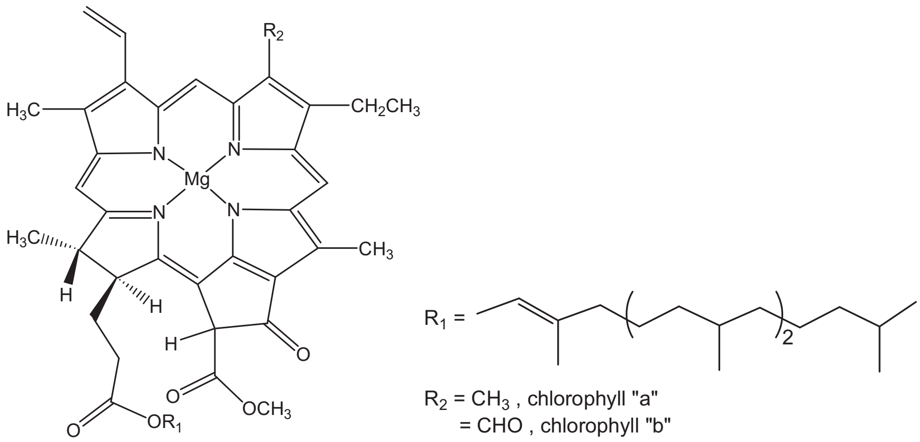


Figure 1. Chemical structures of chlorophyll a and b.

3.1.2. Carotenoids

Carotenoids are naturally occurring pigments that are responsible for the different colours of fruits, vegetables and other plants (Ben-Amotz and Fishler, 1998). Carotenoids are usually yellow to red, isoprenoid polyene pigments derived from lycopene (Figure 2). They are synthesized *de novo* by photosynthetic organisms and some other microorganisms (Borowitzka, 1988). In animals the carotenoids ingested in the diet are accumulated and/or metabolized by the organism, being present in meat, eggs, fish skin (trout, salmon), in the carapace of Crustacea (shrimp, lobster, Antartic krill, crawfish), and in the subcutaneous fat, the skin, the egg yolks, the liver, the integuments, and in the feathers of birds (poultry) (Breithaupt, 2007).

In the algae the carotenoids seem to function primarily as photoprotective agents and as accessory light harvesting pigment, thereby protecting the photosynthetic apparatus against photo damage (Ben-Amotz *et al*., 1987). They also play a role in phototropism and phototaxis (Borowitzka, 1988). Some microalgae can undergo a carotenogenesis process, in response to various environmental and cultural stresses (e.g. light, temperature, salts, nutrients), where the alga stops growth and changes dramatically its carotenoid metabolism, accumulating secondary carotenoids as an adaptation to severe environments (Bhosale, 2004).

The consumption of a diet rich in carotenoids has been epidemiologically correlated with a lower risk for several diseases particularly those in which free radicals are thought to play a role in initiation, such as arteriosclerosis, cataracts, age-related macular degeneration, multiple sclerosis and cancer (Stahl and Sies, 2005; Tapiero et al., 2004). However, unexpected results from intervention studies (ATBC, 1994; Omenn *et al*., 1996) with β-carotene suggest that the threshold between the beneficial and adverse effects of some carotenoids is low and provides a strong stimulus to further understanding the functional effects of specific carotenoids (Van den Berg *et al*., 2000).

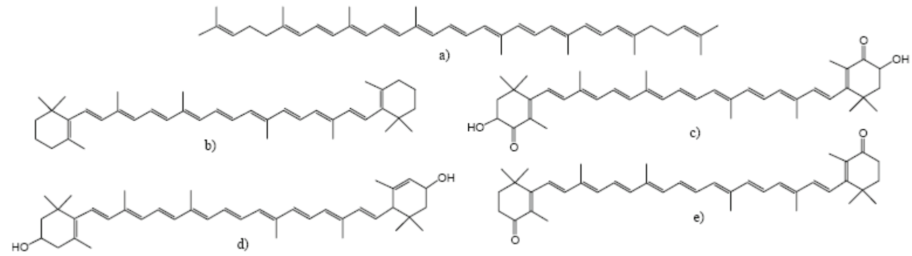


Figure 2. Chemical structures of some carotenoids. a) lycopene, b) □-carotene, c) astaxhanthin, d) lutein, e) canthaxanthin.

More than 600 known carotenoids were reported in nature and about 50 have provitamin-A activity, which includes α-carotene, β-carotene and β-cryptoxanthin (Faure et al., 1999). However, only very few carotenoids are used commercially: β-carotene and astaxanthin and, of lesser importance, lutein, zeaxanthin, lycopene and bixin which are used in animal feeds, pharmaceuticals, cosmetics and food colourings.

The main carotenoids produced by microalgae are β-carotene from *Dunaliella salina* and astaxanthin from *Haematococcus pluvialis*.

β-carotene serves as an essential nutrient and has high demand in the market as a natural food colouring agent, as an additive to cosmetics and also as a health food (Raja *et al*., 2007). β-carotene is routinely used in soft-drinks, cheeses and butter or margarines. Is well regarded as being safe and indeed positive health effects are also ascribed to this carotenoids due to a pro-vitamin A activity (Baker and Gunther, 2004).

The benefits of astaxanthin are said to be numerous, and include enhancing eye health, improving muscle strength and endurance and protecting the skin from premature ageing, inflammation and UVA damage, is a strong coloring agent and has many functions in animals such as growth, vision, reproduction, immune function, and regeneration (Blomhoff *et al*. 1992, Tsuchiya *et al*. 1992, Beckett and Petrovich, 1999). Some reports support the assumption that daily ingestion of astaxanthin may protect body tissues from oxidative damage as this might be a practical and beneficial strategy in health management. It has also been suggested that astaxanthin has a free radical fighting capacity worth 500 times that of vitamin E (Dufossé *et al*., 2005).

3.1.3. Phycobiliproteins

Besides chlorophyll and carotenoid lipophilic pigments, Cyanobacteria (blue-green algae), Rhodophyta (red algae) and Cryptomonads algae contain phycobiliproteins, deep colored water-soluble fluorescent pigments, which are major components of a complex assemblage of photosynthetic light-harvesting antenna pigments - the phycobilisomes (Glazer, 1994). Phycobiliproteins are formed by a protein backbone covalently linked to tetrapyrrole chromophoric prosthetic groups, named phycobilins (Figure 3). The main natural resources of phycobiliproteins are the cyanobacterium *Spirulina* (*Arthrospira*) for phycocyanin (blue) and the rhodophyte *Porphyridium* for phycoerythrin (red).

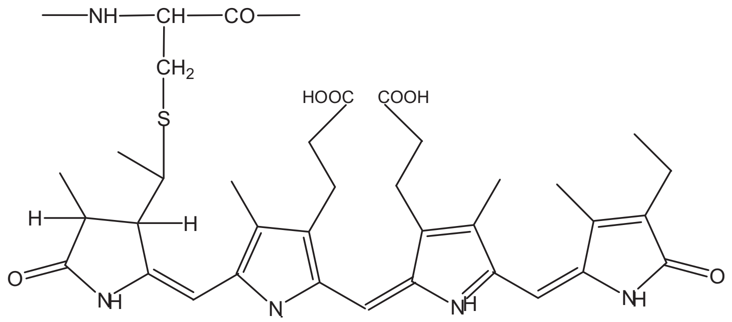


Figure 3. Chemical structure of a phycocyanobilin attached by thioether linkage to the apoprotein.

This group of pigments possesses a large spectrum of applications, which is evidenced by the recent work of Sekar and Chandramohan (2007) that screened 297 patents on phycobiliproteins from global patent databases. They are extensively used for fluorescence applications, as highly sensitive fluorescence markers in clinical diagnosis and for labeling antibodies used in multicolour immunofluorescence or fluorescence-activated cell-sorter analysis (Becker, 1994).

Phycocyanin is currently used in Japan and China as a natural colouring, in food products like chewing gums, candies, dairy products, jellies, ice creams, soft drinks (*e.g.* Pepsi® blue) and also in cosmetics such as lipsticks, eyeliners and eye shadows (Sekar and Chandramohan, 2007). In a recent study, phycocyanin was considered a more versatile blue colorant than gardenia and indigo, providing a bright blue color in jelly gum and coated soft candy, despite its lower stability towards heat and light (Jespersen *et al*., 2005). A rising number of investigations revealed several pharmacological properties attributed to phycocyanin including, antioxidant, anti-inflammatory, neuroprotective and hepatoprotective effects (Romay *et al*. 2003; Benedetti *et al*., 2004; Bhat and Madyastha, 2000).

3.2. Fatty Acids

Some microalgae synthesize fatty acids with particular interest (Figure 4), namely γ-linolenic acid (GLA, 18:3ω6) (*Arthrospira*), arachidonic acid (AA, 20:4ω6) (*Porphyridium*), eicosapentaenoic acid (EPA, 20:5ω3) (*Nannochloropsis*, *Phaeodactylum*, *Nitzschia*, *Isochrysis*, *Diacronema*) and docosahexaenoic acid (DHA, 22:6ω3) (*Crypthecodinium*, *Schizochytrim*) (Bandarra *et al*., 2003, Donato *et al*., 2003, Chini Zittelli *et al*., 1999, Molina Grima *et al*., 2003; Spolaore *et al*., 2006). These long chain polyunsaturated fatty acids (more than 18 carbons) can not be synthesized by higher plants and animals, only by microalgae which supply whole food chains with (Pulz and Gross, 2004). Is estimated that only healthy human adults are able to elongate 18:3ω3 to EPA in an extend lower than 5% and convert EPA to DHA in a rate inferior to 0.05%, being inhibit in childhood and elderly life (Burdge and Calder, 2005, Wang *et al.*, 2006) . This statement confirms the importance of the inclusion of these long chain fatty acids in daily diet.

GLA (18:3ω6)

AA (20:4ω6)

EPA (20:5ω3)

DHA (22:6ω3)

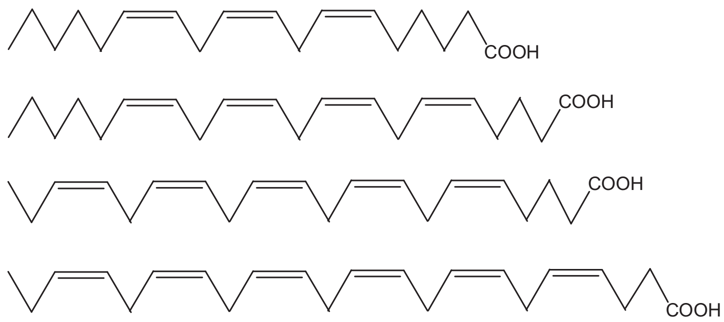


Figure 4. Chemical structure of polyunsaturated fatty acids of high pharmaceutical and nutritional value.

Fish and fish oils are the main sources of LC-PUFA's, still global fish stocks are declining due to general fishing methods and over-fishing and the derived oils are sometimes contaminated with a range of pollutants, heavy metals, toxins and typical fishy smell, unpleasant taste and poor oxidative stability (Certik and Shimizu, 1999, Luiten *et al*., 2003). The production of LC-PUFA from microalgae biotechnology is an alternative approach, and currently microalgal DHA from *Crypthecodinium* and *Ulkenia* is commercially available by the Martek (USA) and Nutrinova (Germany) companies (respectively), for application in infant formulas, nutritional supplements and functional foods (Pulz and Gross, 2004, Spolaore *et al*., 2006).

PUFA's ω-3, especially DHA, are essential in infant nutrition, being important building blocks in brain development, retinal development and ongoing visual, cognitive, as well as important fatty acids in human breast milk (Ghys *et al*., 2002, Wroble *et al*., 2002, Arteburn *et al*., 2007, Crawford, 2000). Long chain *n*-3 fatty acids consumption has been associated with the regulation of eicosanoid production (prostaglandins, prostacyclins, tromboxanes and leucotrienes) which are biologically active substances that influence various functions in cells and tissues (e.g. inflammatory processes) being important in the prophylaxis and therapy of chronic and degenerative diseases including reduction of blood cholesterol, protection against cardiovascular, coronary heart diseases, atherosclerosis, diabetes, hypertension, rheumatoid arthritis, rheumatism, skin diseases, digestive and metabolic diseases as well as cancer (Simopoulos, 2002, Bønaa *et al*., 1990, Sidhu, 2003, Thies *et al*., 2003). Other important role is attributed to gene expression regulation, as well as cholesterol and fasting triacylglycerol (TAG) decreases (Calder, 2004).

The evidence of a dietary deficiency in long-chain omega3 fatty acids is firmly linked to increased morbidity and mortality from coronary heart disease.

.3.3. Tocopherols and Sterols

Tocopherols have a widespread occurrence in nature being present in both photosynthetic (e.g. leaves) and non-photosynthetic (e.g. seedlings) tissues of higher plants and algae. However *Euglena* microalga has the highest tocopherols content among the several genera of yeast, molds and algae tested (Kusmic *et al*., 1999).

Studies covering a wide range of phytoplankton have suggested that the growth rates of bivalves are related to the kind and amount of sterols present in the diet phytoplankton (Wikfors *et al*., 1991). On the other hand, it has been found that many polyhydroxysterols from marine organisms have anticancer, cytotoxic and other biological activity (Cui *et al*., 2000, Tang *et al*., 2002, Han *et al*., 2003, Volkman, 2003).

3.4. Proteins

The high protein content of various microalgae species is one of the main reasons to consider them as an unconventional source of protein (Soletto *et al*., 2005), well illustrated by the great interest in microalgae as single cell protein (SCP) during the 1950s. In addition, the amino acid pattern of almost all algae compares favorably with that of other food proteins. Since the cells are capable of synthesize all amino acids, they can provide the essential ones to humans and animals (Guil-Guerrero *et al*., 2004). As other bioactive compounds synthesized by microalgae, amino acids composition, especially the free amino acids, varies greatly between species as well as with growth conditions and growth phase (Borowitzka, 1988). Protein or amino acids may therefore be by-products of an algal process for the production of other fine chemicals, or with appropriate genetic enhancement, microalgae could produce desirable amino acids in sufficiently high concentrations (Borowitzka, 1988).

3.5. Polysaccharides

Polysaccharides are widely used in the food industry primarily as gelling and/or thickening agents. Many commercially used polysaccharides like agar, alginates and carrageenans are extracted from macroalgae (*e.g. Laminaria*, *Gracilaria*, *Macrocystis*) (Borowitzka, 1988). Nevertheless, most microalgae produce polysaccharides and some of them could have industrial and commercial applications, considering the fast growth rates and the possibility to control the environmental conditions regulating its growth. The most promising microalga for commercially purposes is the unicelular red alga *Porphyridium cruentum*, which produces a sulphated galactan exopolysaccharide that can replace carrageenans in many applications. Another example is *Chlamydomonas mexicana*, which releases up to 25% of its total organic production as extracellular polysaccharides and which as found application as a soil conditioner in the USA (Borowitzka, 1988). Certain highly sulphated algal polysaccharides also present pharmacological properties acting on the stimulation of the human immune system (Pulz and Gross, 2004).

3.6. Vitamins and Minerals

Microalgae biomass represents a valuable source of nearly all essential vitamins (*e.g.* A, B_1, B_2, B_6, B_{12}, C, E, nicotinate, biotin folic acid and pantothenic acid) and a balanced mineral content (*e.g*. Na, K, Ca, Mg, Fe, Zn and trace minerals) (Becker, 2004). The high levels of vitamin B_{12} and Iron in some microalgae, like *Spirulina*, makes them them particularly

suitable as nutritional supplements for vegetarian individuals. The vitamin content of an alga depends on the genotype, the stage in the growth cycle, the nutritional status of the alga, the light intensity (photosynthetic rate). The vitamin content is therefore amenable to manipulation by varying the culture conditions as well as by strain selection or genetic engineering. However, vitamins cell content fluctuates with environmental factors, the harvesting treatment and the biomass drying methods (Brown *et al*., 1999, Borowitzka, 1988).

3.7. Antioxidants

Microalgae are photoautotrophic organisms that are exposed to high oxygen and radical stresses, and consequently have developed several efficient protective systems against reactive oxygen species and free radicals (Pulz and Gross, 2004). Hence, there is increasing interesting in using microalgae as natural antioxidants source for cosmetics (*e.g.* sun-protecting) and functional food/nutraceuticals.

Natrah *et al*. (2007) reported a stronger antioxidant activity exhibited by methanolic microalgal crude extracts (from *e.g. Isochrysis galbana*, *Chlorella vulgaris*, *Nannochloropsis oculata, Tetraselmis tetrathele*, *Chaetoceros calcitrans*) when compared with α-tocopherol, but lower than the synthetic antioxidant BHT. However BHT and BHA synthetic antioxidants, are questionable in terms of their safe use, since they are believed to be carcinogenic and tumorigenic if given in high doses (Schildermann *et al*., 1995, Aruoma, 2003).

3.8. Pharmaceuticals and Other Biologically Active Compounds

The microalgae represent a very large, relatively unexploited reservoir of novel compounds, many of which are likely to show biological activity, presenting unique and interesting structures and functions (Yamaguchi, 1997). In the last decades marine microorganisms, particularly Cyanobacteria, have been screened for new pharmaceuticals and antibiotics. Published data until 1996 revealed 208 cyanobacterial compounds with biological activity while in 2001 the number of compounds screened was raised to 424, including lipoproteins (40%), alkaloids, amides and others (Burja *et al*., 2001). The reported biological activities comprise cytotoxic, antitumor, antibiotic, antimicrobial (antibacterial, antifungal, antiprotozoa), antiviral (*e.g.* anti-HIV) activities as well as biomodulatory effects like immunosuppressive and anti-inflammatory (Burja *et al*., 2001; Singh *et al*., 2003). The cytotoxic activity, important for anticancer drugs development, is likely related to defense strategies in the highly competitive marine environment, since usually only those organisms lacking an immune system are prolific producers of secondary metabolites such as toxins (Burja *et al*., 2001).

4. Microalgae in Animal Nutrition

Several microalgae (*e.g. Chlorella*, *Tetraselmis*, *Spirulina*, *Nannochloropsis*, *Nitzchia*, *Navicula*, *Chaetoceros*, *Scenedesmus*, *Haematococcus*, *Crypthecodinium*), macroalgae (*e.g. Laminaria*, *Gracilaria*, *Ulva*, *Padina*, *Pavonica*) and fungi (*Mortierella*, *Saccharomyces*, *Phaffia*, *Vibrio marinus*) can be used in both terrestrial and aquatic animal feed (Harel and Clayton, 2004).

Feeds can be formulated by using vegetable protein sources, vegetable oil sources, fishmeal, mineral and vitamin premixes in order to reach appropriate nutritional properties for each animal group and promote health and welfare benefits (Harel and Clayton, 2004). Using even very small amounts of microalgal biomass can positively affect the physiology of animals by improved immune response, resulting in growth promotion, disease resistance, antiviral and antibacterial action, improved gut function, probiotic colonization stimulation, as well as by improved feed conversion, reproductive performance and weight control (Harel and Clayton, 2004). The external appearance of the animals may also be improved, resulting in healthy skin and a lustrous coat, for both farming animals (poultry, cows, breeding bulls) and pets (cats, dogs, rabbits, ornamental fishes and birds) (Certik and Shimizu, 1999).

Since feed corresponds to the most important exogenous factor influencing animal health and also the major expense in animal production, the use of alternative high quality protein supplements replacing conventional protein sources is encouraged. Considering that animal feed stands at the beginning of the food chain, increasing public and legislative interest is evident, especially considering intensive breeding conditions and the recent trend to avoid *"chemicals"* like antibiotics (Breithaupt, 2007). The large number of nutritional and toxicological evaluations already conducted has demonstrated the suitability of algae biomass as a valuable feed supplement (Becker, 1994). In fact, 30% of the current world algal production is sold for animal feed applications (Becker, 2004).

4.1. Poultry

The replacement of conventional protein in broilers rations was done by several feeding trials and authors, using various microalgae species, namely *Chlorella*, *Euglena*, *Oocystis*, *Scenedesmus*, *Spirulina*, with incorporation % depending on algae specie (usually up to 10%) (Becker, 1994). In laying hens no differences were found in egg production rate and egg quality (size, weight, shell thickness, solid content of the egg, albumin index, etc) and feed conversion efficiency, between control and birds receiving 12% sewage-growth *Chlorella* (Becker, 1988). Algae may serve as almost the sole source of protein in layers ration (Becker, 1988) and the yolk can have a distinct intense orange colour in layers feed the algal diet (Becker, 2004).

For pigmentation purposes of broilers and/or egg yolks the diet must contain a carotenoids source. Traditionally, dehydrated alfalfa meal and yellow corn were used (Marusich *et al.*, 1960, Becker, 2004). However, today, feed mills use low-cost raw material to provide high energy diets and control the pigment content by appropriate supplementation. Petals of Aztec marigold (*Tagetes erecta*), rich in lutein, have been reported to be very effective as yolk pigmenting agent as well as synthetic canthaxanthin (Madiedo and Sunde, 1964). For laying hens feeds, canthaxanthin should not exceed 8 mg/kg since at extremely

high dosages minute crystals may be formed in the retina by a reversible deposition process (Breithaupt, 2007).

In the last decades, microorganisms such as microalgae, have been tested for pigmentation purposes in poultry. *Dunalliella bardawil* can be a source of vitamin A and a yolk enhancing agent when administrated to laying hens (Avron *et al*., 1952). Gouveia et al. (1996a) reported the effect of carotenoids present in *Chlorella vulgaris* microalga biomass upon pigmentation of egg yolk comparable with commercially synthetic pigments used. *Haematococcus* microalga can also be used as a natural feed colourant of broiler chickens (Kenneth, 1989, Waldenstedt *et al*., 2003).

Studies with chickens fed red microalga *Porphyridium* sp. biomass (at 5% and 10% diet incorporation), showed a reduced blood cholesterol level and a modified fatty acid composition in egg yolk, in spite of no differences in body weight, egg number, and egg weight (Ginzberg *et al*., 2000). Chickens fed with algal biomass consumed 10% less food for both groups, and their serum cholesterol levels were significantly lower (by 11% and 28% for the groups fed with 5% and 10% supplement, respectively) as compared with the respective values of the control group. Egg yolk of chickens fed with algae tended to have reduced cholesterol levels (by 10%) and increased linoleic acid and arachidonic acid levels (by 29% and 24%, respectively). In addition, the color of the egg yolk was darker as a result of the higher carotenoid levels (2.4 fold higher) for chickens that fed with 5% supplement (Ginzberg *et al*., 2000).

Algae are, in general, officially approved in several countries as chicken feed and do not require new testing or approval. However, it has to be decided from case to case how restrictive the different algae species are regarded as feed supplements (Becker, 1994). In the European Union the Regulation (EC) No. 1831/2003 determines the use of additives in animal nutrition and sets out rules for the authorization, marketing and labeling of feed additives.

4.2. Pigs

Aside from poultry, pigs appear to be a potential group for which algae could be used as feed supplement. *Chlorella* and *Scenedesmus* were used for substituting soybean meal and cotton seed meal in concentrations up to 10%, without differences in feed conversion efficiency (Hintz *et al*., 1966, Hintz and Heitmann, 1967). Microalgal biomass is a feed ingredient of good nutritional quality and suited very well for rearing pigs. It can replace conventional proteins like soybean meal or fishmeal and no difficulties in acceptability of algae were reported for these animals (Becker, 1994).

Spirulina has also been tested as additive in short-term and long-term experiences (Fevrier and Seve, 1975) and all parameters studied remained identical and no differences in reproductive capacity were observed. The authors recommended 25% of microalgal biomass incorporation, while Yap *et al*. (1982) assumed 33% incorporation, without negative symptoms.

4.3. Ruminants

It should be expected that ruminants represent the group of animals most suitable for feeding with algae, since these animals are able to digest even unprocessed algal material (*e.g.* cell walls). However a limited number of trials have been done due the large amount of algae required to perform appropriate feeding experiments with these animal species. Sheep's, lambs and cattle's shows an inability to digest efficiently the carbohydrate fraction of the algae (*Chlorella*, *Scenedesmus obliquus* and *Scenedesmus quadricauda*) (Hintz *et al*., 1966, Davis *et al*., 1975). Better digestibility was obtained with *Spirulina* constituting 20% of a complete sheep diet. Calves revealed a minor difference between control and untreated fresh *Scenedesmus* alga feeding animals (Calderon *et al*., 1976).

4.4. Aquaculture

Microalgae feeds are currently used mainly for the culture of larvae and juvenile shell- and finfish, as well as for raising the zooplankton required for feeding of juvenile animals (Benemann, 1992, Chen, 2003). They are required for larval nutrition during a brief period, either for direct consumption in the case of molluscs and peneid shrimp or indirectly as food for the live prey, mainly rotifers, copepods and *Artemia* nauplii, which in turn are used for crustaceans and fish larvae feeding (Brown *et al*., 1997, Duerr *et al*., 1998, Muller-Feuga, 2000, Xu *et al*., 2007).

In 1999, the production of microalgae for aquaculture reached 1000 t (62% for mollusks, 21% for shrimps and 16% for fish) for a global world aquaculture production of 43×10^6 t of plants and animals (Muller-Feuga, 2000). The most frequently used species in aquaculture are *Chlorella*, *Tetraselmis*, *Isochrysis*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*, *Nannochloropsis*, *Skeletonema* and *Thalassiosira* (Yamaguchi, 1997, Borowitzka, 1997, Apt and Behrens, 1999, Muller-Feuga, 2000).

Microalgae contain essential nutrients which determine the quality, survival, growth and resistance to disease of cultured species. These illustrate the importance of the control of microalgal biochemical composition for the success of aquaculture feed chains, opening new perspectives for the study of fish larval nutrition and the development of microalgae-based feeds for aquaculture (Fábregas *et al*., 2001). To support a better balanced nutrition for animal growth, it is often advised to use mixed microalgae cultures, in order to have a good protein profile, adequate vitamin content and high polyunsaturated fatty acids, mainly EPA, AA and DHA, recognized as essential for survival and growth during the early stages of life of many marine animals (Volkman *et al*., 1989). One of the beneficial effects attributed to adding algae is an increase in ingestion rates of food by marine fish larvae which enhance growth and survival as well as the quality of the fry (Naas *et al*. 1992). In addition, the presence of algae in rearing tanks of European sea bass larvae has been shown to increase digestive enzyme secretion (Cahu and Zambonino-Infante 1998).

Aquatic species, such as salmonids (salmon and trout), shrimp, lobster, seabream, goldfish and koi carp under intensive rearing conditions need a supplementation of carotenoids pigments in their diet, to attain their characteristic muscle colour. In addition to pigmenting effects, carotenoids, namely astaxanthin and canthaxanthin, exert benefits on animal health and welfare, promote larval development and provide growth and performance

stimulatory effects in farmed fish and shrimp (Baker and Gunther, 2004). These effects were proved by Torrissen (1984) during the early star-feeding of Atlantic salmon reared in fresh water, by Christiansen *et al*. (1995) in Atlantic salmon parr and by Torrissen and Christiansen (1995) that proposed a minimum of 10 mg astaxanthin or canthaxanthin per kg of diet for all fish and crayfish; also for non-salmonid fish, authors have reported growth benefits with carotenoids supplementation, for instance in carp and tilapia (Segner *et al*., 1989) and in crustacean, such as prawn *Panaeus japonicus* (Chien and Jeng, 1992, Nègre-Sadargues *et al*., 1993).

A positive metabolic role of carotenoids in the nutrition of larval fish and survival of young fry was also discussed by Reitain *et al*. (1997), Shahidi *et al*. (1998), Planas and Cunha (1999) and Lazo *et al*. (2000). However, the inclusion of 45 mg carotenoids in the diet (Rema and Gouveia, 2005) besides this effectiveness on skin pigmentation, was not sufficient to induce any differences in growth and survival of larvae and juvenile goldfish, independently of the source (natural or synthetic).

Nevertheless, given carotenoids high costs, efforts have been deployed to evaluate the potential of some natural pigments obtained from the red yeast *Phaffia rhodozyma* (Bon *et al*., 1997), the marine bacteria *Agrobacterium aurantiacum* (Yokoyama and Miki, 1995), the green algae *Haematococcus pluvialis* (Harker *et al*., 1996, Yuang and Chen, 2000), *Chlorella zofingiensis* (Bar *et al*., 1995) and *Chlorella vulgaris* (Gouveia *et al*., 1996b) as dietary carotenoid sources. Numerous reports show that carotenogenic microalgae appear as suitable source of carotenoids in fish feeds.

Haematococcus pluvialis was assayed in rainbow trout for colouring purposes (Sommer *et al*., 1991, 1992, Choubert and Heinrich, 1993) in spite of less flesh pigmentation than by synthetic astaxanthin, due the esterified form of astaxanthin and a low availability of the pigment inside the alga spore. However, Gomes *et al*. (2002) proved their efficiency on skin pigmentation of gilthead seabream and Gouveia *et al*. (2003, 2005) in ornamental goldfish and koi carp.

Chlorella vulgaris biomass proved to be efficient, comparable with synthetic astaxanthin and canthaxanthin, for pigmentation purposes, in rainbow trout (Gouveia *et al*., 1996c, 1997, 1998), gilthead seabream (Gouveia *et al*., 2002), ornamental goldfish and koi carp (Gouveia *et al*. 2003, 2005) and shrimps (Passos *et al*., in preparation).

Spirulina (rich in β-carotene) is usually used in aquaculture feeds up to 5-20% as a fish and shrimp feed (Benemann, 1992) and to enhances the red and yellow patterns in carp while leaving a brilliant white colour (Gouveia *et al*., 2003, 2005, Spolaore *et al*., 2006) and in ornamental goldfish (Gouveia *et al*., 2003, 2005).

Haslea ostrearia, a diatom, induces a blue-green colour on the gills and labial palps of oysters, which increase market's value by 40% (Spolaore *et al*., 2006).

5. Microalgae in Human Nutrition

In early 1950's microalgae were considered to be a good supplement and/or fortification in diets for malnourished children and adults, as a single cell protein but nowadays microalgae for human nutrition is marketed in different forms of tablets, capsules and liquids (Spolaore *et al*., 2006).

Some nutritional studies were done with humans and the authors suggest that the algae daily consumption should be restricted to about 20 g, with no harmful side effects occur, even after a prolonged period of intake (Becker, 1988). Gross *et al*. (1978) performed a study feeding algae (*Scenedesmus obliquus*) to children (5 g/daily) and adults (10 g/daily), incorporated into their normal diet, during four-week test period. Hematological data, urine, serum protein, uric acid concentration and weight changes were measured, and no changes in the analyzed parameters were found, except a slight increase in weight, especially important for children. The same authors also carried out a study with a slightly (group I) and seriously (group II) malnourished infant during three weeks. The four-years-old children of group I (10 g algae/daily) showed a significant increase in weight (27 g/day) compared with the other children of the same group who received a normal diet, and no adverse symptoms were recorded. The second group was nourished with a diet enriched with 0.87 g algae/kg body weight, substituting only 8% of the total protein and the daily increase in weight was about sevenfold (in spite on a low protein contribution) and all anthropogenic parameters were normal. The authors concluded that the significant improvement in the state of the health was attributed not only to the algal protein but also to therapeutic factors.

However, adults are very resistant in the acceptation of novel foods with microalgae incorporation, which was demonstrated by Feldheim (1972) and Gross and Gross (1978) because it often affects conservative ethnic factors, including religious and socio-economic aspects (Becker, 1994) being much easier with children's who are more willing to accept uncommon preparations. This was demonstrated in Mexico, where a beverage formed by 50% of a suspension of *Spirulina* ("green milk") was given, without problems, as bottle feed to babies (Jacket, 1974).

6. New Trends in Microalgae Food Applications

All over the world commercial production of microalgae for human nutrition is already a reality. Numerous combinations of microalgae or mixtures with other health foods can be found in the market in the form of tablets, powders, capsules, pastilles and liquids, as nutritional supplements (Table 2). They can also be incorporated into food products (*e.g.* pastas, biscuits, bread, snack foods, candies, yoghurts, soft drinks), providing the health-promoting effects that are associated with microalgal biomass, probably related to a general immune-modulating effect (Belay, 1993). In spite of some reluctance for novel foods in the past, nowadays there is an increasing consumer demand for more natural food products, presenting health benefits. Functional foods supplemented with microalgae biomass are sensorily much more convenient and variable, thus combining health benefits with attractiveness to consumers (Pulz and Gross, 2004). In some countries (Germany, France, Japan, USA, China, Thailand), food production and distribution companies have already started serious activities to market functional foods with microalgae and cyanobacteria (Pulz

and Gross, 2004). Food safety regulations for human consumption are the main constraint for the biotechnological exploitation of microalgal resources, but successful cases such as the approval (9 December 2002) of the marine diatom *Odontella aurita* by Innovalg (France) as a novel food, following EC Regulation 258/97, broadens perspectives.

In the last years, our research group in Portugal aims to develop a range of novel attractive healthy foods, prepared from microalgae biomass, rich in carotenoids and polyunsaturated fatty acids with antioxidant effect and other beneficial properties. At the same time toxicological studies involving all the microalgae to be incorporated are also been conducted. Traditional foods, like mayonnaises, gelled desserts, biscuits, pasta and breakfast cereals, largely consumed on daily basis on different European diets, are be used as vehicles to those nutraceuticals. This strategy avoids the hassled of changing food habits; considering that Europeans are getting older and have strong cultural motivations, being highly resistant to food innovations. The impact of natural substances introduced in the diet via "usual" foods is proved to be efficient at long term and do not present the drawbacks of traditional therapeutic actions based on medicines of short term impact.

Table 2. Major microalgae commercialized for human nutrition (Adapted from Pulz and Gross, 2004, Spolaore *et al*., 2006 and Hallmann, 2007)

Microalga	Major Producers	Products	World production (t/year)
Spirulina (*Arthrosphira*)	Hainan Simai Pharmacy Co. (China) Earthrise Nutritionals (California, USA) Cyanotech Corp. (Hawaii, USA) Myanmar Spirulina factory (Myanmar)	powders, extracts tablets, powders, extracts tablets, powders, beverages, extracts tablets, chips, pasta and liquid extract	3000
Chlorella	Taiwan Chlorella Manufacturing Co. (Taiwan) Klötze (Germany)	tablets, powders, nectar, noodles powders	2000
Dunaliella salina	Cognis Nutrition and Health (Australia)	powders β-carotene	1200
Aphanizomenon flos-aquae	Blue Green Foods (USA) Vision (USA)	capsules, crystals powder, capsules, crystals	500

The viability of incorporating microalgal biomass in food systems is conditioned by the applied processing type and intensity (*e.g*. thermal, mechanical), by the nature of the food matrix (*e.g*. emulsion, gel, aerated dough systems) and to the interactions with other food components (*e.g.* proteins, polysaccharides, lipids, sugars, salts). Besides colouring and nutritional purposes, introducing microalgal ingredients in food systems, can also impart significant changes in its microstructure and rheological properties (Batista *et al*., 2006a). These aspects are particularly focused in our research.

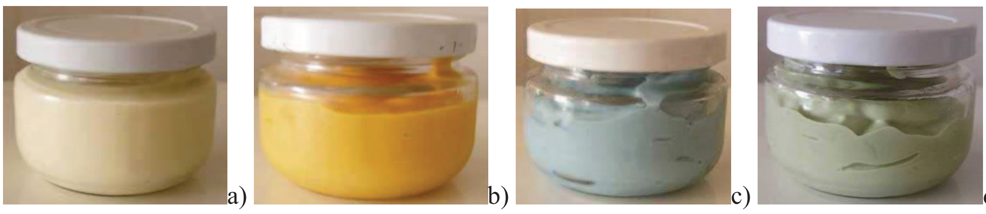
6.1. Oil-in-Water Emulsions

The development of coloured oil-in-water emulsions using natural sources, especially from microalgal origin, is an interesting field to investigate. The attainment of appealing and stable colourations is an important innovation for these types of products. Due to the antioxidant properties that most natural pigments present it is also possible to improve the resistance to oil oxidation, which is particularly advantageous in high fat products like emulsions.

6.1.1. Emulsions Coloured with Natural Pigments

The addition of natural pigments, typically present in microalgae, to oil-in-water (o/w) emulsions was studied by Batista *et al*. (2006a, 2006b). The emulsions were prepared with 3% (w/w) pea protein isolate and 65% (w/w) vegetable oil, according to previous studies that successfully replaced egg yolk protein by leguminous proteins in o/w emulsions (Raymundo *et al*., 2002). Commercial lutein oil dispersion (FloraGlo®, Kemin, USA) and phycocyanin extracted from *Sprirulina* (*Arthrospira*) *maxima* laboratory cultures (INETI, Portugal) (Reis *et al*., 1998) were used, at concentrations ranging from 0.25% to 1.25% (w/w). Emulsions containing both pigments, in different proportions (total pigment concentration of 0.5% w/w) were also prepared. Regarding carotenoids lipophilic character, lutein was added to the emulsions dispersed oil phase while phycocyanin, being an hydrophilic proteinaceous pigment, was added to the continuous aqueous phase, prior to the emulsification process.

Lutein (yellow) and Phycocyanin (blue) imparted appealing and innovative colourations to food emulsions, as can be observed in Figure 5. However, the addition of these pigments had significant implications on the emulsions structural and rheological properties. The effects were markedly different for the two pigments used. Their distribution between the continuous (aqueous) and dispersed (oil) phase and its interactions with the emulsifier molecules at the interface seems to be of major importance (Batista *et al*., 2006b).



a) b) c) d)

Figure 5. Oil-in-water (o/w) pea protein-stabilized emulsions, a) without pigment addition (control), b) with 0.50% (w/w) lutein, c) with 0.50% (w/w) phycocyanin, d) with both pigments in equal proportion 50L:50P (0.50% total piment).

The addition of lutein had a negative impact on the emulsion microstructure and rheological characteristics (Figure 6), although there were no significant differences between samples with different lutein concentrations. Adding lutein to the oil fraction could have modified the nature of the emulsions' dispersed phase, namely the strength of the attractive interactions between molecules and the effectiveness of their packing in the condensed phase (McClemments, 1999). Recent studies (Granger *et al*., 2003, Rampon *et al*., 2004) have suggested that not only the surfactant molecules, *i.e*. emulsifiers and proteins, but also the fat

used in the emulsions formulation participates in the development of the interface characteristics and rheological properties. Lutein molecules are mainly lipophylic molecules but present polar hydroxyl groups in both ends of the conjugated polyisoprenoid chain, so it is possible to interact with hydrophobic domains of the pea protein emulsifier, creating weaker and disordered layers. Santipanichwong and Suphantarika (2007) also reported emulsion destabilization by the addition of lutein in reduced-fat mayonnaises with spent brewers' yeast as fat replacer.

On the other hand, phycocianin addition resulted in a significant improvement of the emulsions rheological properties (Figure 6) which increased linearly with phycocyanin concentration (Batista *et al*., 2006a). The presence of phycocyanin protein molecules may have contributed to a marked increase in the viscosity of the aqueous continuous phase, thus retarding the oil droplet association movements and consequently enhancing emulsion stability. It is also possible that phycocyanin protein molecules interact in the interfacial protein adsorbed layer at the surface of oil droplets, reinforcing in this case the pea protein emulsifier film and imparting stability to emulsions. In fact, previous studies (Chronakis *et al*., 2000) have demonstrated that a protein isolate from blue-green algae (*Spirulina platensis* strain Pacifica), containing phycocyanin, was capable of reducing the interfacial tension at the aqueous/air interface at relatively lower bulk concentrations compared to common food proteins.

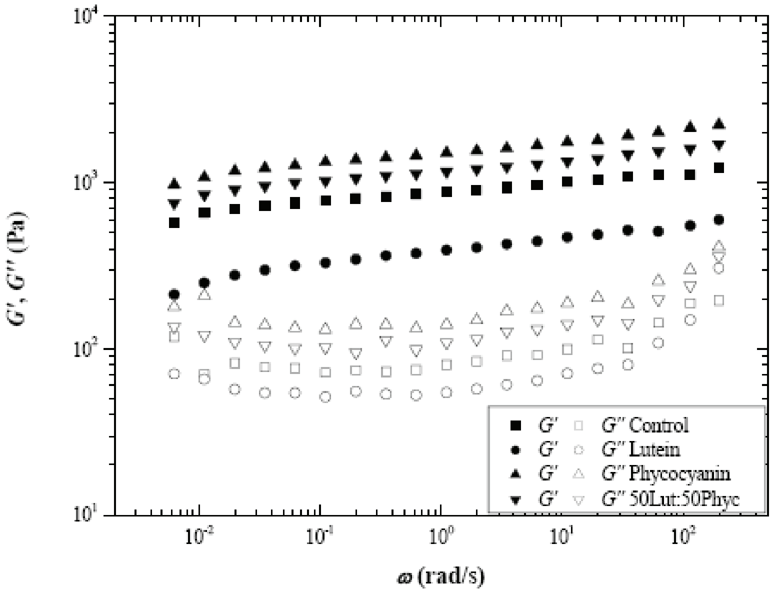


Figure 6. Mechanical spectra of o/w emulsions without pigment addition (control), with 0. 50% lutein, 0.50% phycocyanin, and with both pigments in equal proportion 50L:50P (0.50% total pigment).

When using combinations of both pigments, an increase of the rheological and parameters with phycocyanin proportion was apparent, and a synergetic effect was observed when using small amounts (< 50% proportion) of lutein.

6.1.2. Emulsions Coloured with Microalgal Biomass

The use of the microalgae *Haematococcus pluvialis* (carotenogenic) and *Chlorella vulgaris* (before and after carotenogenesis) to colour oil-in-water pea protein-stabilized emulsions was also investigated by the authors (Gouveia *et al*., 2006), obtaining a wide range of attractive and stable tonalities (Figure 7). These microalgae were cultivated in the Biomass Unit of the Department of Renewable Energies from INETI (Portugal).

Figure 7. Oil-in-water (o/w) pea protein-stabilized emulsion with 0.25%, 0.50% and 0.75% (w/w) (from left to right) of *Haematococcus pluvialis* (top) and *Chlorella vulgaris* biomass (carotenogenic) (bottom).

The colour stability of the emulsions was evaluated, through the evolution of the L*a*b* parameters (CIELAB system) along six weeks. The primary and secondary oxidation products of the emulsions were also determined, and an enhanced resistance to oxidation was evidenced by emulsions containing microalgae (Gouveia *et al*., 2006). The incorporation of *Haematococcus pluvialis* provided higher oxidation stability over time, in comparison with *Chlorella vulgaris.* It should be considered that during carotenogenesis *Haematococcus pluvialis* accumulates mainly astaxanthin while canthaxanthin is the dominant carotenoid in *Chlorella vulgaris*. The higher oxidation stability of astaxanthin as already been reported, and is related to the fact that antioxidant effectiveness of carotenoids increases as the number of the conjugated double bounds of carotenoids increased (Yen and Chen, 1995). However, microalgal biomass may be considered as multi-component antioxidant systems, which are generally more effective due to synergistic or additive interactions between the different antioxidant components.

The addition of microalgal components improved the emulsion textural parameters which should be related with a higher stability level. It can also be observed that 0.75% (w/w) biomass seems to be an optimal concentration level, since the three emulsions presented similar firmness values (2.3-2.5 N) (Figure 8). At higher concentrations the emulsions became excessively firm, which could be related to an increase on the viscosity of the aqueous phase.

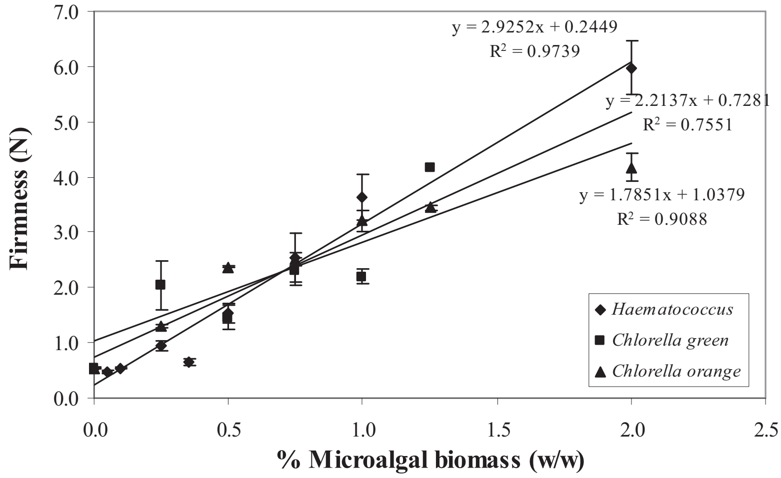


Figure 8. Firmness values of oil-in-water pea protein-stabilized food emulsions coloured with different concentrations of *Haematococcus pluvialis*, *Chlorella vulgaris* green and *Chlorella vulgaris* orange biomass.

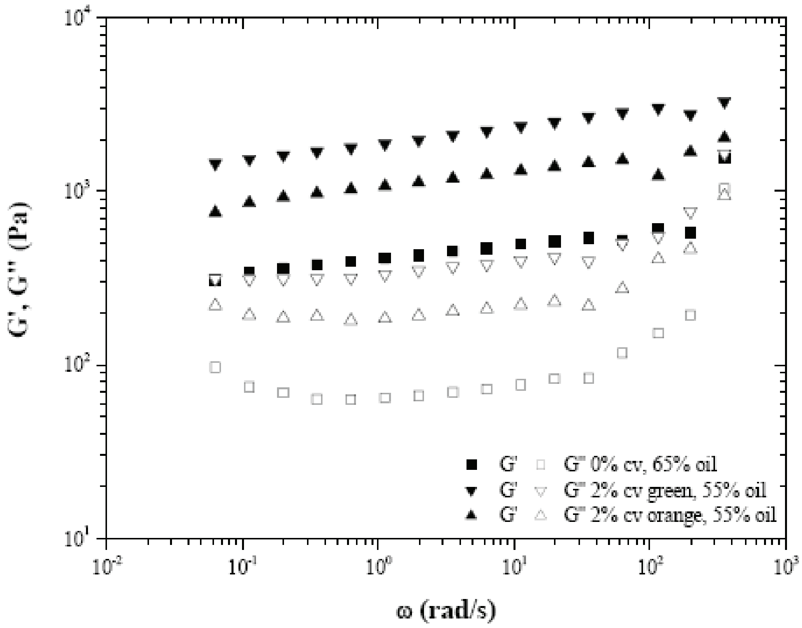


Figure 9. Mechanical spectra of pea protein-stabilized o/w emulsions with and without 2% w/w Chlorella green and orange biomass, at different oil contents.

The capacity of the *Chlorella vulgaris* biomass as a fat mimetic, and its emulsifier ability, has also been studied (Raymundo *et al*., 2005). Pea protein emulsions with *Chlorella vulgaris* addition (both green and orange - carotenogenic) were prepared at different protein (2-5% w/w) and oil (50-65% w/w) contents, characterized in terms of rheological behaviour. It was observed that emulsions with 55% oil and 2% microalga were more structured than the

emulsions with 65% oil and no microalgal biomass addition (Figure 9). This behaviour can be explained by the increase of the viscosity of the continuous phase of the emulsion, by the microalgal material. This result supports the potential use of using microalgae material to act as a fat mimetic, besides the possible advantages as colouring and antioxidant agent. The development of the emulsion structure did not occur when microalgal biomass fully replaced the vegetable protein as an emulsifier, and phase separation was instantaneous.

6.2. Biscuits

Short dough cookies and biscuits are widely consumed food products, appreciated for their taste, versatility, convenience, conservation, texture and appearance. The use of natural ingredients, exhibiting functional properties and providing specific health benefits beyond traditional nutrients, is a very attractive way to design new food products, with an important market niche presently exhibiting pronounced growth.

6.2.1. Biscuits Coloured with Chlorella Vulgaris Biomass

A study was undertaken to determine the effects of adding *Chlorella vulgaris* biomass as a colouring ingredient in traditional butter biscuits (Gouveia *et al*., 2007a). The cookies were manufactured at a pilot scale, according to an optimized formulations from previous studies (Piteira *et al*., 2004), and stored for three months at room temperature, protected from light and air.

Chlorella vulgaris biscuits presented an accentuated green tonality (Figure 10), which increased with the amount of added biomass. In general, colour parameters (CIELAB system) remained very stable along the storage period. However, it seems not necessarily to use biomass concentrations above 1% (w/w), since the green tonality (-a*) differences are no longer significant ($p<0.05$), and higher algal concentrations are related with some colour variations along time (Figure 11a).

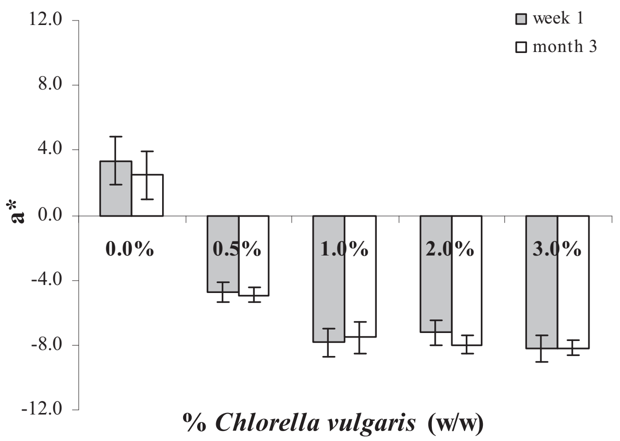

a)


b)

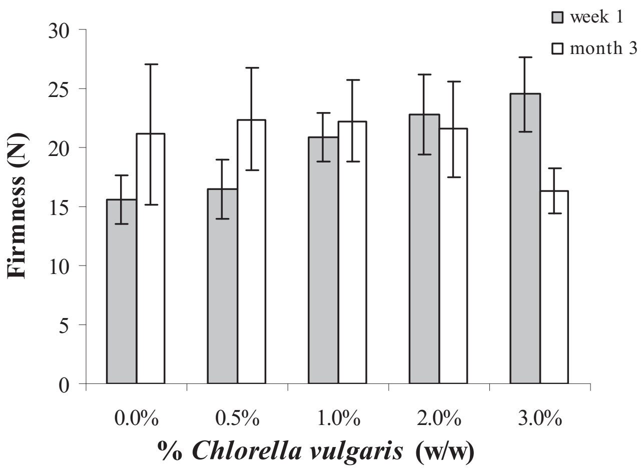
Figure 10. Biscuits with Chlorella vulgaris biomass, a) at various concentration levels (0.0-3.0%), b) and in comparison with Haematococcus pluvialis (pink) and Chlorella vulgaris (orange) carotenogenic biomass addition.

The texture profile of the biscuits was also evaluated, and a significant increase of their firmness was evidenced with an increase of added microalgal biomass (Figure 11b). These

results evidence the positive effect of the alga in the biscuit structure, reinforcing the short dough system. Biscuit are considered solid emulsions of sucrose, lipids and non-gelatinized starch (Hoseney *et al*., 1988), being this morphology is responsible for the biscuits structure and texture. The main factor affecting these properties is the moisture content and water mobility, which are highly affected by the interaction with hydroxyl groups present in the matrix (Hoseney *et al*., 1988). The replacement of a small amount of flour by microalgae biomass, resulted in the inclusion of a complex biomaterial, rich in different proteins and polysaccharides. These molecules have an important role on the water absorption process, which promote the increase of biscuits firmness, resulting in more compact structures.



a)



b)

Figure 11. Green chromaticity a* (a) and firmness values (b) of biscuits with different concentrations of Chlorella vulgaris biomass, after one week and three months storage.

6.2.2. Biscuits with Isochrysis Galbana Biomass Rich in Polyunsaturated Fatty Acids

A similar study was performed, this time using *Isochrysis galbana* biomass (Gouveia *et al*., 2007b), that was cultivated in the Department of Aquaculture of IPIMAR (Portugal). An enhancement of the biscuits texture properties and high stability of colour and texture along three months storage was observed, as previously reported for *Chlorella* biscuits (Gouveia *et al*., 2007a). The biscuits presented quite different tonalities, turning from green to a brownish and duller tonality when increasing the biomass concentration from 1.0% to 3.0% (Figure 12).

Figure 12. Biscuits with different incorporation levels (0.0%, 1.0%, 3.0%) of Isochrysis galbana biomass.

The main interest in using *Isochrysis galbana* biomass is due to its high levels of long chain omega-3 polyunsaturated fatty acids, especially EPA and DHA (Bandarra *et al*., 2003). The biscuits fatty acids profile is clearly related to butter (Özkanli and Kaya, 2007), with predominance of saturated (~60%) and monounsaturated fatty acids (~30%), mainly palmitic acid (30-40%) and oleic acid (18:1ω9) (20-25%), respectively. Polyunsaturated fatty acids corresponded to 6-9% (4-5% linoleic acid; 18:2ω6), the highest levels being for 3% *Ig* biscuits (55% linoleic acid, 15% EPA, 6% α-linolenic acid and 3% DHA).

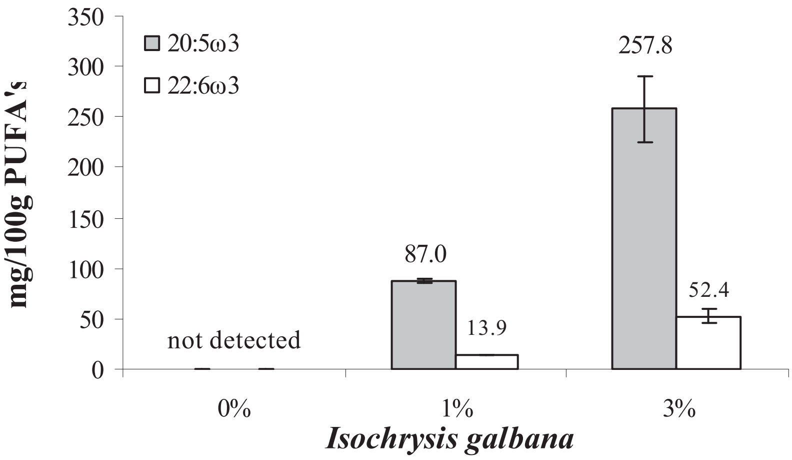


Figure 13. Omega-3 polyunsaturated fatty acids, of biscuits with 0%, 1% and 3% *Isochrysis galbana* biomass incorporation.

In spite of the drastic thermal processing (high temperatures) during biscuits manufacturing, the addition of microalgal biomass leads to the presence of ω3 fatty acids (absent in control biscuits) which remain stable along storage time (Figure 13). The thermal resistance of fatty acids should be due to its presence in an encapsulated form, inside the microalga. Ig biscuits presented LC-PUFA's-ω3 levels (EPA+ DHA) of 100 mg/100g and 30 mg/100g biscuit, for 1% and 3% microalgal biomass incorporation, respectively (Figure 13). These values reflect an important source of PUFA-ω3 with a moderate biscuit consumption, as the recommendations for dietary intake in healthy adults is 500 mg/day (ISSFAL, 2004).

6.3. Food Gels

Most recently, our group is studying the incorporation microalgal biomass in food gelled products, based on protein and polysaccharide mixed biopolymer systems. Gelled vegetable desserts, alternative to dairy desserts, with pea protein isolate (4% w/w), κ-carrageenan (0.15%) and starch (2.5%), optimized in previous studies (Nunes *et al*., 2003, 2006) were used as model systems. The gels were prepared with different microalgae - *Spirulina* (*Arthrosphira*) *maxima*, *Chlorella vulgaris* (green and orange, after carotenogenesis), *Haematococcus pluvialis* (red, carotenogenic) and *Diacronema vlkianum* – were evaluated in terms of colour and texture and compared with gels prepared with commercial pigments – phycocyanin, astaxanthin, β-carotene, canthaxanthin and lutein. The microalgae gels imparted less intense tonalities (Figure 14) and texture modifications, compared to the pigment addition.

The pea protein/κ-carrageenan/starch mixed gel systems with 0.75% (w/w) microalgal biomass addition were characterized in terms of rheological behaviour, including monitoring of the viscoelastic functions (G', G") during gelification (cooling process) and maturation kinetics (Batista *et al*., 2007b).

(a) (b)

Figure 14. Gelled vegetable desserts with incorporation of microalgae biomass (a) and commercial pigments (b).

The incorporation of these biomaterials seemed to be beneficial, especially for *Haematococcus pluvialis* which promoted a structural reinforcement expressed by improved rheological properties (Figure 15). This may be related to its significantly higher fat content

(40.7%) in relation to other microalgae. The influence of fat content on gelling behaviour has been studied in milk gelled systems (Houzé *et al*., 2005, Vélez-Ruiz *et al*., 2005) being concluded that using high fat milk rather than skim milk results in stronger gels, which is usually attributed to fat droplets acting as active filler particles embedded in the protein matrix.

However, the addition of *Spirulina* promoted a drastic reduction on the gels rheological parameters (Figure 15), which should be related with a thermodynamic incompatibility between the microalgal protein and other components of the mixed gelled system. In fact, Chronakis (2001) reported that proteins isolated from *Spirulina* are quite intricate biomaterials, likely to be protein and/or protein-pigment (phycocyanin) complexes rather than individual protein molecules. Therefore, *Spirulina* denaturation and gel formation is a complex phenomenon *per se*, which can probably interfere with the gelling process of the biopolymers present in the mixed gel system.

Further research is required in order to better understand the gelation mechanism of these microalgae and the specific interactions with each biopolymer present in the complex mixed gel system, as well as the influence of processing conditions (e.g. temperature, heating and cooling rates).

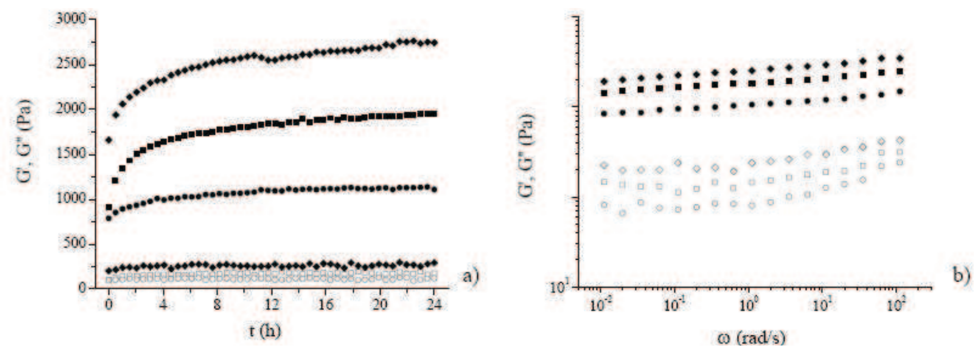


Figure 15. Maturation kinetic curves (a) and mechanical spectra (b), at 5ºC, of pea protein/κ-carrageenan/starch gels (■) with 0.75% (w/w) *Haematococcus pluvialis* (♦) and *Spirulina maxima* (●) biomass. G' (filled symbol), G" (open symbol).

Conclusion

The combination of the exceptional nutritional value of microalgae with colouring and therapeutical properties, associated with an increase demand of natural products, make microalgae worth exploring for utilization in the future in feed, food, cosmetic and pharmaceutical industries, with recognized advantages comparing with the traditional ingredients.

In the actual scenario with multiple pharmacological treatments, many believe that simple dietary interventions or nutritional supplements may be more natural, acceptable and feasible method of providing benefits.

Choose of the right food to eat in an early stage of life associated with a healthy lifestyle can have important benefits in future life. A healthy diet based on microalgae novel food products can have important benefits for all age groups.

The great results obtained by the authors in the preparation of common food products with microalgae incorporation providing attractive and healthier food with enormous potential as a functional food ingredient.

In the near future, the authors' intent to continue the development of healthier food products, preparing other widespread food product, such as pasta, salt crackers, extruded products with the incorporation of microalgal biomass, as a vehicle of functional ingredients, namely pigments, antioxidants and PUFA's.

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References

Aasen, A.J., Eimhjellen, K.E., and Liaaen-Jensen, S. (1969). An extreme source of β-carotene. *Acta Chemica Scandinavica*, *23*, 2544-2545.

Apt, K.E., and Behrens, P.W. (1999). Commercial developments in microalgal biotechnology. *Journal of Phycology*, *35*, 215-226.

Arteburn, L.A., Oken, H.A., Hoffman, J.P., Bailey-Hall, E., Cheng, G., Rom, D., Hamersley, J., and McCarthy, D. Bioequivalence of docosahexaenoic acid from different algal oils in capsules and in DHA-fortified food. *Lipids*, In press (DOI: 10.1007/s11745-007-3098-5).

Aruoma, O.I. (2003). Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. *Mutation Research*, *523*, 9-20.

ATBC Cancer Prevention Study Group (1994). The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *The New England Journal of Medicine*, *330*, 1029-1035.

Avron, M., Ben-Amotz, A., and Edelstein, S. (1952). Use of *Dunalliella bardawil* as a source of vitamin A and a yolk-color enhancing agent. Commonwealth of Australia Patent Act 71864/87.

Bandarra, N.M., Pereira, P.A., Batista, I., and Vilela, M.H. (2003). Fatty acids, sterols and α – tocopherol in *Isochrysis galbana*. *Journal of Food Lipids*, *18*, 25-34.

Baker, R., and Gunther, C. (2004). The role of carotenoids in consumer choice and the likely benefits from their inclusion into products for human consumption. *Trends in Food Science and Technology*, *15*, 484-488.

Balder, HF, Vogel, J., Jansen, M.C., Weijenberg, M.P., van den Brandt, P.A., Westenbrink, S., van der Meer, R., and Goldbohm, R.A. (2006). Heme and chlorophyll intake and risk of colorectal cancer in the Netherlands cohort study. *Cancer Epidemiology Biomarkers and Prevention*, *15*,717-725.

Bar, E., Rise, M., Vishkautsan, M., and Arad, S. (1995). Pigment and structural changes in *Chlorella zofingiensis* upon light and nitrogen stress. *Journal of Plant Physiology*, *146*, 527-534.

Batista, A.P., Raymundo, A., Sousa, I., and Empis, J. (2006a). Rheological characterization of coloured oil-in-water food emulsions with lutein and phycocyanin added to the oil and aqueous phases. *Food Hydrocolloids*, *20*, 44-52.

Batista, A.P., Raymundo, A., Sousa, I., Empis, J., and Franco, J.M. (2006b). Colored food emulsions – implications of pigment addition on the rheological behaviour and microstructure. *Food Biophysics*, *1*, 216-227.

Batista, A.P., Bandarra, N., Raymundo, A., and Gouveia, L. (2007a). Microalgae biomass – a potential ingredient for the food industry. *EFFoST/EHED Joint Conference*. Lisbon, Portugal.

Batista, A.P., Gouveia, L., Nunes, M.C., Franco, J.M., and Raymundo, A. (2007b). Microalgae biomass as a novel functional ingredient in mixed gel systems. In Gums *and Stabilisers for the Food Industry* – 14th Edition. Eds. P.A. Williams, G.O. Phillips. Royal Society of Chemistry.

Becker, E.W. (1988). Micro-algae for human and animal consumption. In M.M. Borowitzka, and L.J. Borowitzka (Eds), *Micro-algal biotechnology* (pp. 222-256). Cambridge University Press.

Becker, E.W. (1994). *Microalgae: biotechnology and microbiology*. Cambridge University Press.

Becker, E.W. (2004). Microalgae in human and animal nutrition. In A. Richmond (Ed), *Handbook of microalgal culture* (pp. 312-351). Oxford: Blackwell.

Beckett, B.R., and Petkovich, M. (1999). Evolutionary conservation in retinoid signalling and metabolism. *American Zoology*, *39*, 783-795.

Belay, A. (1993). Current knowledge on potential health benefits of *Spirulina platensis*. *Journal of Applied Phycology*, *5*, 235-240.

Ben-Amotz, A., and Avron, M. (1980). Glicerol, β-carotene and dry algal meal production by commercial cultivation of *Dunaliella*. In G. Shelef, and C.J. Soeder (Eds), *Algae Biomass* (pp. 603-610). Amsterdam: Elsevier/North Holland Biomedical Press.

Ben-Amotz, A., Gressel, J., and Avron, M. (1987). Massive accumulation of phytoene induced by norflurazon in *Dunaliella bardawil* (Chlorophyceae) prevents recovery from photoinhibition. *Journal of Phycology*, *23*, 176-181.

Ben-Amotz, A., and Fishler, R. (1998). Analysis of carotenoids with emphasis on 9-*cis*-β-carotene in vegetables and fruits commonly consumed in Israel. *Food Chemistry*, *62*, 515-520.

Benedetti, S., Benvenuti, F., Pagliarani, S., Francogli, S., Scoglio, S., and Canestrari, F. (2004). Antioxidant properties of a novel phycocyanin extract from the blue-green alga *Aphanizomenon flos-aquae. Life Sciences*, *55*, 2353-2362.

Benemann, J.R. (1992). Microalgae aquaculture feeds. *Journal of Applied Phycology*, *4*, 233-245.

Bhat, V.B., and Madyastha, K.M. (2000). C-Phycocyanin: a potent peroxyl radical scavenger in vivo and in vitro. *Biochemical and Biophysical Research Communications*, *275*, 20-25.

Bhosale, P. (2004). Environmental and cultural stimulants in the production of carotenoids from microorganisms. *Applied Microbiology and Biotechnology*, *63*, 351-361.

Blomhoff, R., Green, M.H., and Norum, K.R. (1992). Vitamin A: physiological and biochemical processing. *Annual Reviews in Nutrition*, *12*, 37-57.

Bon, J.A., Leathers, T.D., and Jayaswal, R.K. (1997). Isolation of astaxanthin-overproducing mutants of *Phaffia rhodozyma*. *Biotechnology Letters*, *19*, 109-112.

Bønaa, K.H., Bjerve, K.S., Straume, B., Gram, I.T., and Thelle, D. (1990) Effect of eicosapentaenoic and docosahexaenoic acids on blood pressure in hypertension: a population-based intervention trial from the Tromsø study. *New England Journal of Medicine*, *322*, 795-801.

Borowitzka, M.A. (1988). Vitamins and fine chemicals from micro-algae. In M.A. Borowitzka, and L.J. Borowitzka (Eds), *Micro-algal biotechnology* (pp. 153-196). Cambridge, UK: Cambridge University Press.

Borowitzka, M.A. (1997). Microalgae for aquaculture: opportunities and constraints. *Journal of applied phycology*, *9*, 393-401.

Borowitzka, M.A. (1999). Commercial production of microalgae: ponds, tanks, tubes and fermenters. *Journal of Biotechnology*, *70*, 313-321.

Breithaupt, D.E. (2007). Modern application of xanthophylls in animal feeding - a review. *Trends in Food Science and Technology*, *18*, 501-506.

Brown, M.R., Jeffrey, S.W., Volkman, J.K., and Dunstan, G.A. (1997). Nutritional properties of microalgae for mariculture. *Aquaculture*, *151*, 315-331.

Brown, M.R., Mular, M., Miller, I., Farmer, C., and Trenerry, C. (1999). The vitamin content of microalgae used in aquaculture. *Journal of Applied Phycology*, *11*, 247-255.

Burdge, G.C., and Calder, P.C. (2005). alfa-Linolenic acid metabolism in adult humans:the effects of gender and age on conversion to longer-chain polyunsaturated fatty acids. *European Journal of Lipid Science and Technology*, *107*, 426–439.

Burja, A.M., Banaigs, B., Abou-Mansour, E., Burgess, J.G., Wright, P.C. (2001). Marine cyanobacteria - a prolific source of natural products. *Tetrahedron*, *57*, 9347-9377.

Cahu, C.L., and Zambonino-Infante, J.L. (1998). Algal addition in sea bass (*Dicentrarchus labrax*) larvae rearing: effect on digestive enzymes. *Aquaculture*, *161*, 479-489.

Calder, P.C. (2004). Review - n–3 Fatty acids and cardiovascular disease: evidence explained and mechanisms explored. *Clinical Science*, *107*, 1–11.

Calderon, C.J.F., Merino, Z.H., and Barragán, M.D. (1976). Valor alimentico del alga espirulina (*Spirulina geitleri*) para ruminants. *Tecnica Pecuaria en Mexico*, *31*, 42-46.

Campbell, C.J. (1997). *The Coming Oil Crisis*. Essex, England: Multi-Science Publishing Company and Petroconsultants.

Certik, M., and Shimizu, S. (1999). Biosíntesis and regulation of microbial polyunsaturated fatty acid production. *Journal of Biosciences and Bioengineering*, *87*, 1-14.

Chaumont, D. (1993). Biotechnology of algal biomass production: a review of systems for outdoor mass culture. *Journal of Applied Phycology*, *5*, 593-604.

Chen, Y.-C. (2003). Immobilized *Isochrysis galbana* (Haptophyta) for long-term storage and applications for feed and water quality control in clam (*Meretrix lusoria*) cultures. *Journal of Applied Phycology*, *15*, 439-444.

Chien, Y.H., and Jeng, S.C. (1992). Pigmentation of kuruma prawn, *Panaeus japonicus* Bate, by various pigment sources and levels and feeding regimes. *Aquaculture*, *102*, 333-346.

Chini Zittelli, G., Lavista, F., Bastianini, A., Rodolfi, L., Vincenzini, M., and Tredici, M.R. (1999). Production of eicosapentaenoic acid by *Nannochloropsis* sp. cultures in outdoor tubular photobioreactors. *Journal of Biotechnology*, *70*, 299-312.

Choubert, G., and Heinrich, O. (1993) Carotenoid-pigments of the green-alga *Haematococcus pluvialis* – assay on rainbow trout, *Oncorhynchus mykiss*, pigmentation in comparison with synthetic astaxanthin and canthaxanthin. *Aquaculture*, *112*, 217-226.

Christiansen, R., Glette, J., Lie, O., Torrissen, O.J., and Waagbo, R. (1995). Antioxidant status and immunity in Atlantic salmon, *Salmo salar*, fed semi-purified diets with and without astaxanthin supplementation. *Journal of Fish Diseases*, *18*, 317-328.

Chronakis, I.S., Galatanu, A.N., Nylander, T., and Lindman, B. (2000). The behaviour of protein preparations from blue-green algae (*Spirulina platensis* strain Pacifica) at the air/water interface. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *173*, 181-192.

Chronakis, I.S. (2001). Gelation of edible blue-green algae protein isolate (*Spirulina platensis* strain Pacifica): thermal transitions, rheological properties, and molecular forces involved. *Journal of Agricultural and Food Chemistry*, *49*, 888-898.

Clark, D.C., Wilde, P.J., Wilson, D.R., and Wustneck, R. (1992). The interaction of sucrose esters with β-lactoglobulin and α-casein from bovine milk. *Food Hydrocolloids*, *6*, 173-186.

Crawford, M. (2000). Placental delivery of arachidonic acid and docosahexaenoic acids: implication of the lipid nutrition of preterm infants. *American Journal of Clinical Nutrition*, *71*, 275S-284S.

Cui, J.G., Wang, Y., Lin, G.W., and Zeng, L.M. (2000). Polyhydroxylated sterols with biological activities in marine organism. *Natural Products Research Developments*, *12*, 94-100.

Davis, I.F., Sharkey, M.J., and Williams, D. (1975). Utilization of sewage algae in association with paper in diets of sheep. *Agriculture and Environment*, *2*, 333-338.

Donato, M., Vilela, M.H., and Bandarra, N.M. (2003). Fatty acids, sterols, α-tocopherol and total carotenoids composition of *Diacronema vlkianum*. *Journal of Food Lipids*, *10*, 267-276.

Duerr, E.O., Molnar, A., and Sato, V. (1998). Cultured microalgae as aquaculture feeds. *Journal of Marine Biotechnology*, *75*, 65-70.

Dufossé, L., Galaup, P., Yaron, A., Arad, S.M., Blanc, P., Murthy, K.N.C., and Ravishankar, G.A. (2005). Microorganisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality?. *Trends in Food Science and Technology*, *16*, 389-406.

Dutta, D., De, D., Chaudhuri, S., and Bhattacharya, S.K. (2005). Hydrogen production by Cyanobacteria. *Microbial Cell Factories*, *4*, 36-46.

Fábregas, J., Otero, A., Dominguez, A., and Patino, M. (2001). Growth rate of the microalga *Tetraselmis suecica* changes the biochemical composition of *Artemia* species. *Marine Biotechnology*, *3*, 256-263.

Faure, H., Fayol, V., Galabert, C., Grolier, P., Moel, G.L., Steghens, J., Kappel, A.V., Nabet, F. (1999). Carotenoids: 1. Metabolism and physiology. *Annales de Biologie Clinique*, *57*, 169-183.

Feldheim, W. (1972). Studies on use of microalgae in human nutrition. 1. Nutrition tests with algae containing diets in Thailand. *International Journal for Vitamin and Nutrition Research*, *42*, 600-606. Cited by Becker, E.W. (1988).

Ferruzi, M.G., and Blakeslee, J. (2007). Digestion, absorption, and cancer preventive activity of dietary chlorophyll derivatives. *Nutrition Research*, *27*, 1-12.

Fevrier, C., and Seve, B. (1975). Incorporation of *Spirulina maxima* in pig diets. *Annales de la Nutrition et de l'Alimentation*, *29*, 625-650.

Fidalgo, J.P., Cid, A.T.E., Sukenik, A., and Herrero, C. (1998). Effects of nitrogen source and growth phase on proximate biochemical composition, lipid classes and fatty acid profile of the marine microalgae *Isochrysis galbana. Aquaculture*, *166*, 105-116.

Ghys, A., Bakkere, E., Hornstra, G., Van der Hout, M. (2002). Red blood cell and plasma phospholipid arachidonic and docosahexaenoic acid levels at birth and cognitive development at 4 years of age. *Early Human Development*, *69*, 83-90.

Ginzberg, A., Cohen, M., Sod-Moriah, U., Shany, S., Rosenshtrauch, A., and Arad, S. (2000). Chickens fed with biomass of the red microalga *Porphyridium* sp. have reduced blood cholesterol level and modified fatty acid composition in egg yolk. *Journal of Applied Phycology*, *12*, 325-330.

Glazer, A.N. (1994). Phycobiliproteins - a family of valuable, widely used fluorophores. *Journal of Applied Phycology*, *6*, 105-112.

Gomes, E., Dias, J., Silva, P., Valente, L., Empis, J., Gouveia, L., Bowen, J., and Young, A. (2002). Utilization of natural and synthetic sources of carotenoids in the skin pigmentation of gilthead seabream (*Sparus aurata*). *European Food Research and Technology*, *214*, 287–293.

Gouveia, L., Veloso, V., Reis, A., Fernandes, H.L., Empis, J., and Novais, J.M. (1996a). *Chlorella vulgaris* used to colour egg yolk. *Journal of the Science of Food and Agriculture*, *70*, 167–172.

Gouveia, L., Veloso, V., Reis, A., Fernandes, H.L., Empis, J., and Novais, J.M. (1996b). Evolution of pigment composition in *Chlorella vulgaris*. *Bioresource Technology*, *57*, 157-163.

Gouveia, L., Gomes, E., and Empis, J. (1996c). Potential use of a microalgal (*Chlorella vulgaris*) in the pigmentation of rainbow trout (*Oncorhynchus mykiss*) muscle. *Zeitschrift fur Lebensmittel Untersuchung und Forschung*, *202*, 75-79.

Gouveia, L., Gomes, E., and Empis, J. (1997) Use of *Chlorella vulgaris* in rainbow trout, *Oncorhynchus mykiss*, diets to enhance muscle pigmentation. *Journal of Applied Aquaculture*, *7*, 61–70.

Gouveia, L., Choubert, G., Gomes, E., Rema, P., and Empis, J. (1998). Use of *Chlorella vulgaris* as a carotenoid source for salmonids: Effect of dietary lipid content on pigmentation, digestibility and muscular retention. *Aquaculture International*, *6*, 269–279.

Gouveia, L., Choubert, G., Gomes, E., Pereira, N., Santinha, J., and Empis, J. (2002). Pigmentation of gilthead seabream, *Sparus aurata* (Lin 1875), using *Chlorella vulgaris* (Chlorophyta, Volvocales) microalga. *Aquaculture. Research*, *33*, 987-993.

Gouveia, L., Rema, P., Pereira, O., and Empis, J. (2003). Colouring ornamental fish (*Cyprinus carpio* and *Carassius auratus*) with microalgal biomass. *Aquaculture Nutrition*, *9*, 123–129.

Gouveia, L., and Rema, P. (2005). Effect of microalgal biomass concentration and temperature on ornamental goldfish (*Carassius auratus*) skin pigmentation. *Aquaculture Nutrition*, *11*, 19-23.

Gouveia, L., Batista, A.P., Raymundo, A., Sousa, I., and Empis, J. (2006). *Chlorella vulgaris* and *Haematococcus pluvialis* biomass as colouring and antioxidant in food emulsions. *European Food Research and Technology*, *222*, 362-367.

Gouveia, L., Batista, A.P., Miranda, A., Empis, J., and Raymundo, A. (2007a). *Chlorella vulgaris* biomass used as colouring source in traditional butter cookies. *Innovative Food Science and Emerging Technologies*, *8*, 433-436.

Gouveia, L., Coutinho, C., Mendonça, E., Batista, A.P., Sousa, I., Bandarra, N.M., and Raymundo, A. (2007b). Sweet biscuits with Isochrysis galbana microalga biomass as a functional ingredient. *Journal of the Science of Food and Agriculture*, *In press*.

Granger, C., Barey, P., Combe, N., Veschambre, P., and Cansell, M. (2003). Influence of the fat characteristics on the physicochemical behaviour of oil-in-water emulsions based on milk proteins-glycerol esters mixtures. *Colloids and Surfaces B: Biointerfaces*, *32*, 353-363.

Gross, R., Gross, U., Ramirez, A., Cuadra, K., Collazos, C., and Feldheim, W. (1978). Nutritional tests with green *Scenedesmus* with health and malnourished children. *Archiv fur Hydrobiologie, Beihefte Ergebnisse der Limnologie*, *11*, 161-173.

Gross, U., and Gross, R. (1978). Acceptance and product selection of food fortified with the microalga *Scenedesmus*. *Archiv fur Hydrobiologie, Beihefte Ergebnisse der Limnologie*, *11*, 174-183.

Guill-Guerrero, J.L., Navarro-Juárez, R., López-Martínez, J.C., Campra-Madrid, P., Rebolloso-Fuentes, M.M. (2004). Functional properties of the biomass of the three microalgal species. *Journal of Food Engineering*, *65*, 511-517.

Hallmann, A. (2007). Algal transgenics and biotechnology. *Transgenic Plant Journal*, *1*, 81-98.

Han, L., Cui, J.G., and Huang, C.S. (2003). Bioactive polyhydroxy sterols and their sapogenins from marine organisms. *Acta Chimica Sinica*, *23*, 305-311.

Harel, M., and Clayton, D. (2004). *Feed formulation for terrestrial and aquatic animals*. US Patent 20070082008 (WO/2004/080196).

Harker, M., Tsavalos, A.J., and Young, A.J. (1996). Autotrophic growth and carotenoid production of *Haematococcus pluvialis* in a 30 liter air-lift photobioreactor. *Journal of Fermentation and Bioengineering*, *82*, 113-118.

Hintz, H.F., and Heitmann, H. (1967). Sewage-grown algae as a protein supplement for swine. *Animal Production*, *9*, 135-141.

Hintz, H.F., Heitmann, H., Weird, W.C., Torell, D.T., and Meyer, J.H. (1966). Nutritive value of algae grown on sewage. *Journal of Animal Science*, *25*, 675-681.

Hoseney, R.C., Wade, P., and Finley, J.W. (1988). Soft wheat products. In Y. Pomeranz (Ed), *Wheat Chemistry and Technology*, volume II, 3rd edition (pp. 407-456). American Association of Cereal Chemists Monograph Series.

Houzé, G., Cases, E., Colas, B., and Cayot, P. (2005). Viscoelastic properties of acid milk gel as affected by fat content at low level. *Interational Dairy Journal*, *15*, 1006-1016.

International Society for the Study of Fatty Acids and Lipids (ISSFAL) (2004). *Recommendations for dietary intake of polyunsaturated fatty acids in healthy adults*.

Jacket, J. (1974). Utilisations biologiques des *Spirulines. Bulletin de l'Académie veterinaire de France*, *47*, 133-143.

Jespersen, L., Strømdahl, L.D., Olsen, K., and Skibsted, L.H. (2005). Heat and light stability of three natural blue colorants for use in confectionery and beverages. *European Food Research and Technology*, *220*, 261–266

Kato, T. (1994). Blue pigment from *Spirulina. New Food Industry*, *29*, 17-21.

Kenneth, G.S. (1989). Pigmentation supplements for animal feed compositions. United States Patent, nº 4.871.551.

Kusmic, C., Barsacchi, R., Barsanti, L., Gualteri, P., and Passarelli, V. (1999). *Euglena gracilis* as a source of the antioxidant vitamin E. Effects of culture conditions in the wild strain and in the natural mutant WZSL. *Journal of Applied Phycology*, *10*, 555-559.

Lazo, P., Dinis, M.T., Holt, J., Faulk, C., and Arnold, C. (2000). Co-feeding microparticulate diets with algae: toward eliminating the need of zooplankton at first feeding in larval red drum (*Sciaenops ocellatus*). *Aquaculture*, *188*, 339-351.

Liu, C.P., and Lin, L.P. (2001). Ultrastructural study and lipid formation of *Isochrysis* sp. CCMP1324. *Botanical Bulletin Academia Sinica*, *42*, 207-214.

Lorenz, R.T., and Cysewski, G.R. (2000). Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. *Trends in Biotechnology*, *18*, 160-167.

Luiten, E.E.M., Akkerman, I., Koulman, A., Kamersmans, P., Reith, H., Barbosa, M.J., Sipkema, D., and Wijffels, R.H. (2003). Realizing the promises of marine biotechnology. *Biomolecular Engineering*, *20*, 429-439.

Madiedo, G., and Sunde, M. (1964). The effect of algae, dried lake weed, alfalfa and ethoxyquin on yolk colour. *Poultry Science*, *43*, 1056-1061.

Marusich, W.L., De Ritter, E., and Bauerfeind, J.C. (1960). Evaluation of carotenoid pigments for colouring egg yolks. *Poultry Science*, *40*, 1338-1345.

McClements, D.J. (1999). *Food Emulsions: Principles, Practice and Techniques*. CRC Press.

Molina Grima, E., Belarbi, E.H., Acién Fernández, F.G. Robles Medina, A., and Chisti, Y. (2003). Recovery of microalgal biomass and metabolites: process options and economics. *Biotechnology Advances*, *20*, 491-515.

Muller-Feuga, A. (2000). The role of microalgae in aquaculture: situation and trends. *Journal of Applied Phycology*, *12*, 527-534.

Naas, K.E., Naess, T., and Harboe, T. (1992). Enhanced 1st feeding of Halibut Larvae (*Hippoglossus hippoglossus* L) in green water. *Aquaculture*, *105*, 143-156.

Natrah, F., Yosoff, F.M. Shariff, M., Abas, F., and Mariana, N.S. Screening of Malaysian indigenous microalgae for antioxidant properties and nutritional value. *Journal of Applied Phycology*, In press (DOI 10.1007/s10811-007-9192-5).

Nègre-Sadargues, G., Castillo, R., Petit, H., Sancé, S., Martinez, R.G., Milicua, J.C., Choubert, G., and Trilles, J.P. (1993). Utilization of synthetic carotenoids by the prawn *Panaeus japonicus* reared under laboratory conditions. *Aquaculture*, *110*, 151-159.

Nunes, M.C., Batista, P., Raymundo, A., Alves, M.M., and Sousa, I. (2003). Vegetable proteins and milk puddings. *Colloids and Surfaces B: Biointerfaces*, *31*, 21-29.

Nunes, M.C., Raymundo, A., and Sousa, I. (2006). Rheological behaviour and microstructure of pea protein / κ–carrageenan / starch gels with different setting conditions. *Food Hydrocolloids*, *20*, 106-113.

Olaizola, M. (2003). Commercial development of microalgal biotechnology: from the test tube to the marketplace. *Biomolecular Engineering*, *20*, 459-466.

Omenn, G.S., Goodman, G.E., Thornquist, M.D., Balmes, J., Cullen, M.R., Glass, A., Keogh, J.P., Meyskens, F.L., Valanis, B., Williams, J.H., Barnhart, S., Cherniack, M.G., Brodkin, C.A., and Hammar, S. (1996). Risk factors for lung cancer and for intervention effects in CARET, the beta-carotene and retinol efficacy trial. *Journal of the National Cancer Institute*, *88*, 1550-1559.

Oren, A. (2005). A hundred years of *Dunaliella* research: 1905-2005. *Saline Systems*, *1*, 2.

Ötles, S., and Pire, R. (2001). Fatty acid composition of *Chlorella* and *Spirulina* microalgae species. *Journal of AOAC International*, *84*, 1708-1714.

Özkanli, O., and Kaya, A. (2007). Storage stability of butter oils from sheep's non-pasteurized and pasteurized milk. *Food Chemistry*, *100*, 1026-1031.

Passos, R., Lagreze, F., Moriel, D., Bonfim, T., Gouveia, L., Maraschin, M. and Beirão, L. (2007). Pigmentation of pacific white shrimp (*Litopenaeus vannamei*, Boone, 1931) with carotenoids from natural sources (in preparation).

Piteira, F., Nunes, M.C., Raymundo, A., and Sousa, I. (2004). Effect of principal ingredients on quality of cookies with dietary fibre. In P.A. Williams, and G.O. Phillips (Eds), *Gums and Stabilisers for the Food Industry 12* (pp. 475-483). Cambridge, UK: Royal Society of Chemistry.

Planas, M., and Cunha, I. (1999). Larviculture of marine fish: problems and perspectives. *Aquaculture*, *177*, 171-190.

Ponis, E., Probert, I., Véron, B., Mathieu, M., and Robert, R. (2006). New microalgae for the Pacific oyster *Crassostrea gigas* larvae. *Aquaculture*, 253, 618-627.

Pulz, O., and Gross, W. (2004). Valuable products from biotechnology of microalgae. *Applied Microbiology and Biotechnology*, *65*, 635-648.

Radmer, R.J., and Parker, B.C. (1994). Commercial applications of algae: opportunities and constraints. *Journal of Applied Phycology*, *6*, 93-98.

Rampon, V., Brossard, C., Mouhous-Riou, N., Bousseau, B., Llamas, G., and Genot, C. (2004). The nature of the apolar phase influences the structure of the protein emulsifier in oil-in-water emulsions stabilized by bovine serum albumin. A front-surface fluorescence study. *Advances in Colloid and Interface Science*, *108-109*, 87-94.

Raja, R., Hemaiswarya, S., and Rengasamy, R. (2007). Exploitation of *Dunaliella* for β-carotene production. *Applied Microbiology and Biotechnology*, *74*, 517-523.

Raymundo, A., Franco, J.M., Empis, J., and Sousa, I. (2002). Optimization of the composition of low-fat oil-in-water emulsions stabilized by white lupin protein. *Journal of the American Oil Chemists Society*, *79*, 783-790.

Raymundo, A., Gouveia, L., Batista, A.P., Empis, J., and Sousa, I. (2005). Fat mimetic capacity of *Chlorella vulgaris* biomass in oil-in-water food emulsions stabilised by pea protein. *Food Research International*, *38*, 961-965.

Regulation (EC) No 1831/2003 of the European Parliament and the Council of 22 September 2003 on additives for use in animal nutrition. *Official Journal of the European Union*, L268/29,18.10.2003.

Regulation (EC) No 258/1997 of the European Parliament and the Council of 27 January 1997 on novel foods and food ingredients. *Official Journal of the European Union*, L043,14.02.1997.

Reis, A., Mendes, A., Lobo-Fernandes, H., Empis, J.A., and Maggiolly-Novais, J. (1998). Production, extraction and purification of phycobiliproteins from *Nostoc* sp. *Bioresource Technology*, *66*, 181-187.

Reitain, K., Rainuzzo, J.R., Oie, G., and Olsen, Y. (1997). A review of the nutritional effects of algae in marine fish larvae. *Aquaculture*, *155*, 207-221.

Rema, P., and Gouveia, L. (2005). Effect of various sources of carotenoids on survival and growth of goldfish (*Carassius auratus*) larvae and juvenile. *Journal of Animal and Veterinary Advances*, *4*, 654-658.

Reinehr, C.O., and Costa, J.A. (2006). Repeated batch cultivation of the microalga *Spirulina platensis*. *World Journal of Microbiology and Biotechnology*, *22*, 937-943.

Romay, C.H., Gonzalez, R., Ledon, N., Remirez, D., and Rimbau, V. (2003). Phycocyanin: a biliprotein with antioxidant, anti-inflammatory and neuroprotective effects. *Current Protein and Peptide Science*, *4*, 207-216.

Santipanichwong, R. and Suphantarika, M. (2007). Carotenoids as colorants in reduced-fat mayonnaise containing spent brewer's yeast b-glucan as a fat replacer. *Food Hydrocolloids*, *21*, 565-574.

Schilderman, P.A.E.L., ten Vaarwerk, F.J., Lutgerink, J.T., Van der Wurff, A., ten Hoor, F., and Kleinjans, J.C.S. (1995). Induction of oxidative DNA damage and early lesions in rat gastro-intestinal epithelium in relation to prostaglandin H synthese-mediated metabolism of butylated hydroxyanisole. *Food and Chemical Toxicology, 33*, 99-109.

Segner, H., Arend, P., Von Poeppinghausen, K., and Schmidt, H. (1989). The effect of feeding astaxanthin to *Oreochromis* nioloticus and *Colisa* labiosa on the histology of the liver. *Aquaculture*, *79*, 381-390.

Sekar, S., and Chandramohan, M. (2007). Phycobiliproteins as a commodity: trends in applied research, patents and commercialization. *Journal of Applied Phycology*, *In Press* (doi: 10.1007/s10811-007-9188-1).

Shahidi, F., Metusalach, J., and Brown, J.A. (1998). Carotenoids pigments in seafoods and aquaculture. *Critical Reviews in Food Science and Nutrition*, *38*, 1-67.

Shimamatsu, H. (2004). Mass production of *Spirulina*, an edible microalga. *Hydrobiologia*, *512*, 39-44.

Sidhu, K.S. (2003). Health benefits and potential risks related to consumption of fish or fish oil. *Regulatory Toxicology and Pharmacology*, *38*, 336-344.

Simopoulos, A.P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomedicine and Pharmacotherapy*, *56*, 365-379.

Singh, S., Kate, B.N., and Banerjee, U.C. (2005). Bioactive compounds from Cyanobacteria and Microalgae: na overview. *Critical Reviews in Biotechnology*, *25*, 73-95.

Soletto, D., Binaghi, L., Lodi, A., Carvalho, J.C.M., and Converti, A. (2005). Batch and fed-batch cultivations of *Spirulina platensis* using ammonium sulphate and urea as nitrogen sources. *Aquaculture*, *243*, 217-224.

Sommer, T.R., Potts, W.T., and Morrissy, N.M. (1991). Utilization of microalgal astaxanthin by rainbow trout. *Aquaculture*, *94*, 79-88.

Sommer, T.R., Da Souza, F.M.L., and Morrisy, N.M. (1992). Pigmentation of adult rainbow trout, *Oncorhynchus mykiss*, using the green alga *Haematococcus pluvialis*. *Aquaculture*, *106*, 63-74.

Spolaore, P., Joannis-Cassan, C., Duran, E., and Isambert, A. (2006). Commercial applications of Microalgae- review. *Journal of Bioscience and Bioengineering*, *101*, 87-96.

Stahl, W.; Sies, H. (2005). Bioactivity and protective effects of natural carotenoids. *Biochimica et Biophysica Acta*, *1740*, 101-107.

Tang, H.F., Yi, Y.H., and Yao, X.S. (2002). Progress in the study of marine steroids. *Journal of Marine Drugs* (Chinese), *3*, 38-47.

Tapiero, H.; Townsend, D.M.; Tew, K.D. (2004). The role of carotenoids in the prevention of human pathologies. *Biomedicine and Pharmacotherapy*, *58*, 100-110.

Thies, F., Garry, J.M., Yagoob, P., Rerkasem, K., Williams, J., Shearman, C.P., Gallagher, P.J., Calder, P.C., and Grimble, R.F. (2003). Association of n-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: a randomized controlled trial. *Lancet*, *361*, 477-485.

Torrissen, O.J. (1984). Pigmentation of salmonids-effect of carotenoids in eggs and star-feeding diet on survival and growth rate. *Aquaculture*, *4*3, 185-193.

Torrissen, O.J., and Christiansen, R. (1995). Requirements of carotenoids in fish diets. *Journal of Ichthylogy*, *11*, 225-230.

Tsuchiya, M., Scita, G., Freisleben, H.L., Kagan, V.E., and Packer, L. (1992). Antioxidant radical-scavenging activity of carotenoids and etinoids compared to β-tocopherol. *Methods of Enzymology*, *213*, 460 – 472.

Van den Berg, H, Faulks, R., Granado, H.F., Hirschberg, J., Olmedilla, B., Sandmann, G., Southon, S., and Stahl, W. (2000). The potential for the improvement of carotenoid levels in foods and the likely systemic effects. *Journal of the Science of Food and Agriculture*, *80*, 880-912.

Vélez-Ruiz, J.F., González-Tomás, L., and Costell, E. (2005). Rheology of dairy custard model systems: influence of milk-fat and hydrocolloid type. *European Food Research and Technology*, *221*, 342-347.

Volkman, J.K., Jeffery, S.W., Nichols, P.D., Rogers, G.I., and Garland, C.D. (1989). Fatty acid and lipid composition of 10 species of microalgae used in mariculture. *Journal of Experimental Marine Biology and Ecology*, *128*, 219-240.

Volkman, J.K. (2003). Sterols in microrganisms. *Applied Microbiology and Biotechnology*, *60*, 495-506.

Waldenstedt, L., Inborr, J., Hansson, I., and Elwinger, K. (2003). Effects of astaxanthin-rich algal meal (*Haematococcus pluvialis*) on growth performance, caecal campylobacter and clostridial counts and tissue astaxanthin concentration of broiler chickens. *Animal Feed Science and Technology*, *18*, 119-132.

Wang, C., Harris, W.S., Chung, M., Lichtenstein, A.H., Balk, E.M., Kupelnick, B., Jordan, H.S., and Lau, J. (2006). n-3 Fatty acids from fish or fish-oil supplements, but not a-linolenic acid, benefit cardiovascular disease outcomes in primary and secondary prevention studies: a systematic review. *American Journal of Clinical Nutrition*, *84*, 5–17.

Wikfors, G.H., Gladu, P.K., and Patterson, G.W. (1991). In search of the ideal diet for oysters: recent progress with emphasis on sterols. *Journal of Shellfish Research*, *10*, 292-301.

Wikfors, G.H., Ferris, G.E., and Smith, B.C. (1992). The relationship between gross biochemical composition of cultured algal foods and growth of the hard clam, *Mercenaria mercenaria* (L.). *Aquaculture*, *108*, 135-154.

Wroble, M., Mash, C., Williams, L., and McCall, R.B. (2002). Should long chain polyunsaturated fatty acids be added to infant formula to promote development? *Applied Developments in Phychology*, *23*, 99-112.

Xu, Z., Yan, X., Pei, L, Luo, Q., and Xu, J. Changes in fatty acids and sterols during batch growth of *Pavlova viridis* in photobioreactor. *Journal of Applied Phycology*, In press (DOI: 10.1007/s10811-007-9230-3).

Yamaguchi, K. (1997). Recent advances in microalgal bioscience in Japan, with special reference to utilization of biomass and metabolites: a review. *Journal of Applied Phycology*, *8*, 487-502.

Yap, T.N., Wu, J.F., Pond, W.G., and Krook, L. (1982). Feasibility of feeding *Spirulina maxima*, *Arthrospira platensis* or *Chlorella* sp. to pigs weaned to a dry diet at 4 to 8 days of age. *Nutritional Reports International, 35*, 543-552.

Yen, W.J., and Chen, B.H. (1995). Isolation of xanthophylls from Taiwanese orange peels and their effects on the oxidation stability of soybean oil. *Food Chemistry*, *53*, 417-425.

Yokoyama, A., and Miki, W. (1995). Composition and presumed bio-synthetic pathway of carotenoids in the astaxanthin-producing bacterium *Agrobacterium aurantiacum*. *FEMS Microbiology Letters*, *128*, 139-144.

Yuang, J.P., Chen, F. (2000). Purification of trans-astaxanthin from a high-yielding astaxanthin ester-producing strain of the microalga *Haematococcus pluvialis*. *Food Chemistry*, *68*, 443-448.