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Original/*Alimentos funcionales*

Functional characters evaluation of biscuits sublimated with pure phycocyanin isolated from *Spirulina* and *Spirulina* biomass

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Abstract

The aim of the present work is to study the effect of incorporation of biomass and phycocyanin extracts of *Spirulina platensis* growing in define media at large scales (300 liters, limited in nitrogen and high salinity) to traditional butter biscuits in order to increase general mental health as functional products, FPs). The FP were manufactured at a pilot scale formulated by adding algal biomass (0.3, 0.6 and 0.9%) and *S. platensis* phycocyanin (at 0.3%) to wheat flour and stored for one month at room temperature, protected from light and air. The approximate and nutrition composition of *S. platensis* biomass showed high quantity (% dry weight, dw.) of phycocyanin (13.51%, natural food colorant), tocopherols (0.43%), carotenoids (2.65%), vitamins C (1.25%), ω-6, ω-3 fatty acids, essential elements (Fe, Zn, Cr, Se, and others) and antioxidant compounds includes: total phenolic (1.73%), flavonoids (0.87%) and glutathione (0.245 mM). FPs showed a high oxidative stability during storage (30 days) periods (as assessed by antiradical scavenging activity of DPPH and TBA test), compared with that in untreated food products (control). Data of sensory evaluation revealed that FPs containing *S. platensis* biomass or algae extracts were significantly acceptable as control for main sensory characteristics (colour, odour/ aroma, flavor, texture, the global appreciation and overall acceptability). *S. platensis* FPs presented an accentuated green tonality, which increase with the quantity of added biomass. Thus, it could be concluded that functional biscuits had good sensory and nutritional profiles and can be developed as new niche food market.

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EVALUACIÓN DE LAS CARACTERÍSTICAS FUNCIONALES DE GALLETAS SUBLIMADAS CON FICOCIANINA PURA AISLADA A PARTIR DE ESPIRULINA Y BIOMASA DE ESPIRULINA

Resumen

El objetivo del presente trabajo es el estudio del efecto de la incorporación de biomasa y extractos de ficocianina de *Spirulina platensis* cultivados en un entorno definido a gran escala (300 litros, limitado en nitrógeno y alta salinidad) en galletas de mantequilla tradicionales para aumentar la salud mental general con productos funcionales, PF). Los PF fueron elaborados con una formulación a escala piloto añadiendo biomasa de algas (0,3, 0,6 y 0,9%) y *S. platensis ficocianina* (al 0,3%) a la harina de trigo y después se almacenaron durante un mes a temperatura ambiente, protegidos de la luz y del aire. La composición aproximativa y nutricional de la biomasa de *S. platensis* mostró una elevada cantidad (% peso seco, dw.) de ficocianina (13,51%, colorante alimentario natural), tocoferoles (0,43%), carotenoides (2,65%), vitamina C (1,25%), -6, -3 ácidos grasos, elementos esenciales (Fe, Zn, Cr, Se, y otros), así como de compuestos antioxidantes, a saber, fenólico (1,73%), flavonoides (0,87%) y glutationa (0,245 mM) total. Los PF mostraron una alta estabilidad oxidativa durante los periodos de almacenamiento (30 días) (según la evaluación mediante actividad antirradical de pruebas DPPH y TBA), en comparación con la de los productos alimentarios no tratados (control). Los datos de evaluación sensorial revelaron que los PF que contienen biomasa *S. platensis* o extractos de algas fueron significativamente aceptables como control para las características sensoriales principales (color, olor/ aroma, sabor, textura, apreciación global y aceptabilidad global). Los PF *S. platensis* presentaron una acentuada tonalidad verde, que aumenta con la cantidad de biomasa añadida. Así, se podría concluir que las galletas funcionales presentan buenos perfiles sensoriales y nutritivos y que se podrían desarrollar como un nuevo nicho del mercado de la alimentación.

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Introduction

Functional food is considered to be any food or food component that provides health benefits beyond basic nutrition. A great deal of interest has been paid by the consumers towards natural bioactive compounds as functional ingredients in the diets due to their various health beneficial effects[1,2]. However, the term of functional foods (FFs) was considered to be a tool to promote health and well-being for human and animals. Diplock et al.[3] define FFs as a food compounds have positively affect one or more physiological functions (anticarcinogenicity, antimutagenicity, antioxidative and antiaging actions), that could lead to increasing the well-being and/or to reduce the risk of suffering a disease by modulating physiological systems[4]. Therefore, increasing concerns for health, efforts have been made by food industries to develop new functional foods. Modern food industry produces cheap, healthy and more convenient products, in response to increasingly demand consumers. However, among all the food markets, functional foods have been mainly launched in the dairy, confectionery, soft-drinks, bakery and baby-food market[5]. Rapid progress has been made in the development of functional foods based on the results of studies made on food ingredients of microalgae phytochemicals, which that provide positive health benefits. New functional food products launched in the global food and drinks market have followed the route of fortification or addition of desirable microalgae nutrients and its bioactive compounds. The microalgae provides several benefits which include; good sources of healthy oil, essential fatty acids and omega 3,6-fatty acids, high protein quality with good array of amino acids, sulphated polysaccharides, energy, minerals (Se, Zn, Ca, Fe, P), vitamins (vit C, E, folic acid, B 12) zinc and calcium, pigments (carotenoids and phycocyanin), flavonoids and phenolic acids[2,6,7]. However, a variety of biological function of microalgae, which possess antibacterial, antifungal, antiviral, anti-genotoxic, anti-inflammatory, antiulcerogenic, cardioprotective, anti-allergic, anticancer, chemopreventive, antioxidant, hepatoprotective, hypoglycemic and antidiabetic properties have to be taken into consideration[8,9]. Also, some microalgae have been used as colorants for food, and feeding of livestock for meat and fish productions.

In general, microalgae can produce a great variety of secondary metabolites, which do not occur in other organisms[8,10]. The fundamental advantage of using microalgae for industrial production of valuable food ingredients depends on the fact that, for the majority of the species, cultivation is easy and growth is fast[4,11]. However, microalgae can be grown under certain controlled environmental conditions (e.g. temperature, salinity, light, nutrients) that can stimulate or inhibit the biosynthesis and the accumulation of specific bioactive food ingredients (e.g. phycocyanin, astaxanthin and β-carotene) in large quantity[10,12,13]. Additionally, algae considered as bioactive compounds resource, so it is desirable option for fortification[2]. This is research was carried out to establish the bioactive and nutritive compounds present in *Spirulina platensis* biomass and demonstrate health benefits to consumers.

Materials and methods

Reagents

All reagents and chemicals used in the experiments were purchased from Sigma-Aldrich Chemicals. All solvent used were of analytical grade.

Cultivation of algal cells

The *Spirulina platensis* was cultivated (in National Research Centre, Egypt) in 300 liters of Zarrouk's medium[4] containing normal concentrations of NaCl (0.10 M) and low sodium nitrate as a nitrogen source (0.50 g L^{-1}). Aeration was accomplished using air pumps to achieve an air flow rate of 20 L/h. The cultures were gassed with 0.03% volume CO_2 in air and temperature maintained at 25ºC ±3. The pH of all media was adjusted to 9.5. The cultures were illuminated with continuous 10 cool white fluorescent lamps (Philips 40 W) provided an illumination of 2500 lux. In all cultivated aquarium (300 L), conductivity, salinity, pH and temperature were daily measured with Hanna (HI 09812-5) conductivity meter. The purity of cultures was checked periodically by microscopic observation following taxonomy guidelines. All solutions and glassware were autoclaved at 121ºC for 15 min prior to use.

Growth measurements and harvesting

The growth rate of *Spirulina platensis* was monitored every three days through cultivation period by determining the dry weight (dw.) and optical density at 670 nm methods Vonshak[15]. The cells were harvested at the stationary phase, by centrifugation at 10,000 x*g* (4°C) for 15 min and the biomasses were stored at -20°C until analysis.

Separation of phycobiliproteins

Fresh algae (200 g) biomass were added to 2 L of 0.05 M phosphate buffer (pH 6.7) and kept in the dark at 4°C for 12 h, then clarified by centrifugation at 10,000 x*g* (at 4°C) for 15 min. The blue supernatant was decanted and 100 ml of 25% M ammonium sulfate was added and the mixture was left in the dark for 12 h at 4°C. The blue pigment proteins (C-PC crude extract) were precipitated by 50 ml 60% $(NH4)_2SO_4$, after 6 h at 4ºC, C-PC were collected by centrifugation at 10,000 xg for 15 min and the above steps were repeated till a

colorless supernatant was obtained. Then, the protein pellets containing blue pigments were suspended in phosphate buffer and final volume was recorded[4].

Determination of Phycocyanin

The absorption of phycocyanin containing supernatant was spectrophotometrically determined at different wavelengths (620, 652 and 562 nm). The concentrations of phycocyanin (C-PC), allophycocyanin (APC), and phycoerthrin (PE) were deduced using the following formula[16]:

C-PC (mg mL^{-1}) = [*A620* nm – 0.474 *(A652*) nm] / 5.34,
APC (mg mL^{-1}) = [*A652* nm – 0.208 (*A620*) nm] / 5.09,
PE (mg mL^{-1}) = [*A562* nm – 2.41(PC) -0.849 (APC)] / 9.62

Mineral analysis

The minerals were analyzed after acid mineralization in microwave digestion system and dissolved in de-ionized water to standard volume. The concentration of Ca, Cr, P, Mn, Mg, Cu, K, Fe, Zn and Se were determined by using Inductively Coupled Plasma (ICP-AES, Thermo Sci, model: ICP6000 series). Argon gas was used for excitation the element atom. The blank values for each element were deduced from the sample values.

Determination of algal cells total carbohydrates

Total carbohydrates were estimated by the phenol/sulfuric acid colorimetric method[17].

Determination of algal cells total protein

Total nitrogen was determined by using kjeldatherm, Gerhardt laboratory instrument. After acid digestion, ammonium distillation under steam current, and titration with 0.1 N HCl. Total protein was calculated by multiplying total nitrogen by the conventional conversion factor of 6.25[18].

Preparation of carotenoid extracts

The total carotenoids were extracted from algae biomass (10 g) with 100 ml of tetrahydrofuran, in the presence of 10 mg a mixture consists of BHT and magnesium carbonate at ambient temperature for 24 h. Ten ml of the pigment extract was filtered (with 0.45 µm Teflon membrane) and concentrated to about 2 mL by vacuum at 40°C. After complete remove of the solvent with a stream of nitrogen gas, 20 mL of 10% methanolic KOH at room temperature for 2 h was added for saponification. Then, the mixture was transferred to a separator funnel, extracted with 50 mL dichloromethane. The solvent layer was separated, washed several times with distilled water and dried over Na_2SO_4. Then, the solvent was completely removed by nitrogen gas. The total carotenoids obtained were stored under nitrogen at -20°C, until further use.

Determination of algal total carotenoids

The total carotenoids were spectrophotometriclly estimated at 450 nm according to AOAC methods[18]**.** Standard of β-carotene was used for preparing the calibration curve.

Extraction and determination of algal total lipids contents

Total lipids of algae biomass were extracted with a mixture of chloroform: methanol (1:1, v/v), in a Soxhlet apparatus. The extracts were dried under a stream of nitrogen, the resulting residue was used to calculate the total lipids, gravimetrically and expressed as dry weigh percentage.

Identification of fatty acids

The fatty acids of *S. platensis* lipids were analyzed by an HP 6890 series as chromatograph system with an HP 5973 Network mass selective detector. The system was equipped with a TR-FAME (Mass spectroscopy, 30 m, 0.25 mm (70%- cyanopropyl-polysil phenylene) capillary column, with a film thickness of 0.25 µm), injector and transfer line temperatures were 250°C and 240°C, respectively. The oven temperature was programmed as follows: initial temperature; 80°C for 2 min, increase 3°C/min up to 220°C, and then hold at 220°C for 20 min. The carrier gas was He_2 (at rate 1.2 ml/min). The amount of sample injected was about l µl (about 2 mg/ml) and the ionization energy was 70 eV. Qualitative identification of the different fatty acids were performed comparison their relative retention times and mass spectra with those of authentic reference compounds or by comparison of their retention indices and mass spectra with those shown in the NIST (2010) MS spectra. The relative amounts (RA) of individual components of the fatty acids were expressed as percentages of the peak area relative to the total peak areas[19].

Extraction of total phenolic and falvonoid contents

The algae biomass were harvested by continuous flow centrifugation at 2000 xg for 30 min at 4ºC and then the resulting whole cell pellet was weighed. Four grams of pellet were re-suspended in ethanol (20 mL), sonicated to disrupt cells and homogenized for 3 min at

4ºC. The homogenate was centrifuged at 2000 xg for 15 min (at 40ºC), the resulting supernatant was centrifuged again (2000 xg for 10 min). Then, the supernatant was filtered through Millipore filter (0.45 µm pore size), and the filtrate was evaporated till dryness to give a crude algal ethanolic extracts (enrich in phenolic compounds).

Determination of total phenolic compounds

Total phenolic compounds (TPC) of algal extract were spectrophotometrically determined using Folin-Ciocalteau reagent as described by Singleton et al.[20]. A standard calibration curve was prepared using gallic acid.

Determination of falvonoids content

The total flavonoid content (TFC) was estimated spectrophotometercally by the aluminum chloride method based on the formation of complex flavonoid-aluminum[21]. One milliliter of sample was mixed with 1 mL of $AlCl_3$ methanolic solution (2%, w/v). After incubation at room temperature for 15 min, the absorbance was read at 430 nm. The amount of TFC was estimated from the standard calibration curve of 10-100 mg ml^{-1}quercetin.

Determination of total tocopherols

Total tocopherols of algal cells were spectrophotometrically determined as described by Wong et al.[22].

Extraction and determination of ascorbic acid

Ascorbic acid (vitamin C) was extracted from the cells with 2% metaphosphoric acid, and determined by spectrophotometric methods using 2,6 di-chlorophenol indophenol dye[23].

Determination of glutathione (GSH)

The GSH content of algal cell extracts was measured by reaction with 5,5'dithiobis- 2-nitrobenzoic (DTNB) reagent to give a compound that absorbed at 412 nm[24]. Concentration of GSH was expressed as µM.

Preparation of Biscuits supplemented with Spirulina platensis *cells*

The food function products (FFP, biscuits) were prepared using 46.5% flour, 23% sugar, 20% butter, 10% water and 0.5% of baking powder. Algal biomass was incorporated into FFP at 0.3%, 0.6 and 0.9 % concentration levels (w/w). Phycocyanin as active algae ingredient was added at 3% level to FFP, and kept as standard control. A control food product, without any food additive, was also prepared. All FFP were baked in an oven (Freibol, FB Model) at 125°C during 35 min. After preparation, the FFP were stored inside plastic bags, in sealed glass container, at room temperature and protected from light.

Antioxidant activity of functional food products (FFP) during storage time

The antioxidant activity of FFP was measured by the scavenging ability of DPPH radical and reducing power methods. All measurement were replicated 3 times and averaged.

1. DPPH scavenging radical assay

The ability of the functional biscuits samples to scavenge DPPH radical was estimated according to the method of Tagashira and Ohtake[25]. The radical- scavenging activity was calculated from a calibration curve. The concentration providing 50% inhibition (IC_{50}) was calculated from a graph representing the inhibition percentage against PC concentration.

2. Determination of lipids oxidation products

The products of lipids oxidation of FFP was estimated based on thiobarbituric acid (TBA) reactivity method. Samples were evaluated for malondialdehyde (MDA) production using a spectrophotometric assay for TBA[26]. The extinction coefficient at 532 nm of 153 Mcm^{-1} for the chromospheres was used to calculate the concentration of MDA-like TBA produced (mM).

Sensory evaluation of functional biscuits

A twenty member of un-trained panel comprising of staff and researchers from the Plant Biochemistry and Food Sciences and Nutrition Departments (National Research Centre) was asked to mark the scores of main sensory characteristics (colour, odour/aroma, flavour, texture and the global appreciation) of biscuits samples prepared with different amount of algae cells or main compounds extracts. Participants were informed about the study and explained that their participation was entirely voluntary, that they could stop the interview at any point and that the responses would be anonymous. Also, this study has been done in accordance with the National Research Centre Ethics Committee, Egypt. Evaluation of the biscuits was conducted for 24 hours after baking. The panelists were used the points hedonic scale method: 9 (excellent) to 1 (very poor).

Sensory testing was done on all samples in triplicates. Samples were prepared according to good hygiene and manufacturing practices each panelist was presented with coded randomized samples. Each sample was coded with three random three digit numbers and the positions of the samples were randomized. Panelists were seated in individual sensory booths and given distilled water to neutralize their mouth between the samples. The score were statistically analyzed by ANOV test.

Statistical analysis

Data were analyzed with SPSS version 11.0 (Illinois, USA) using one-way Analyses of variance (ANOVA). The significance differences were tested using the Duncan Multiple Range test. Three replications were used for chemical and physical analysis and two replications for sensory evaluation (n=20).

Results and Dissection

Chemical composition of Spirulina platensis *cells*

S. platensis microalgae was selected after preliminary studies to cultivated at large scales (in 300 liters medium), in medium contained low nitrogen source (0.5 g/L sodium nitrate) coupled high salinity (0.1 M NaCl) in order to enhance the accumulation of physiological function molecules[4]. The results revealed that *S. platensis* had high quality proteins, oil rich with unsaturated fatty acids, carbohydrates, phycocyanin and carotenoids (as photosynthetic pigments), and antioxidant compounds (include: total phenolic, flavonoids, tocopherols, ascorbic acid and glutathione). As shown in table I, *S. maxima* contained high quantity of proteins, oils and carbohydrates with values 40.57%, 20.40% and 16.32%, respectively. Phytothensytic pigments, phycocyanin (13.51%) and carotenoids (2.51%) were found in significant amounts. The concentration of antioxidant compounds including: phenolic, flavonoids, tocopherols, ascorbic acid and glutathione in algae biomass were found to be 1.73%, 0.87%, 0.43%, 1.25% and 0.245 mM, respectively. The results is in agreement with the finding of Abd El Baky and El-Baroty[4], that the high amounts of phycocyanin and antioxidant compounds was obtained in *S. maxima* cultivated under salt stress[10,27]. However, the literature has established that in microalgae general the content of lipids is less than 4%[6]. The lipid content of the *S. platensis* in this work was 20.20 g/100 g (dw.), this value is significantly higher than those determined in many microalgae species by Abd El Baky and El Baroty[4,10]. However, the enhance production of lipids and pigments in microalgae could be as a result of the growth condition and nutrient composition such as N_2 and NaCl concentration[4]. In this study also the *S. platensis* contained high contents of important of proteins, lipo-soluble and hydro-soluble vitamins, antioxidant compounds and carbohydrates over that found in traditional vegetables and fruits. Thus, it may be considered a potential dietary food to provide significant proportions of proteins, vitamins and carbohydrates requirements for human. Abd El Baky and El Baroty[10] reported that the phycocyanin, carotenoids and phenolic compounds present in some microalgae species had high ability as an antioxidant, enhancer the immune system and as an anti-inflammatory agent.

Based on ICP-analysis, algae biomass was found to containing high concentrations of essential elements include: P, K, Ca, Mg, Cr, Cu, Fe, Mn, Se and Zn (Table II). The values of these elements were 340.63 mg/100g biomass, 0.28 mg/100g, 0.45 mg/100g, 0.74

Table I
Chemical composition of Spirulina platensis *grown in a culture contain high salt and low nitrogen concentration*

Compounds	*Content*[a]
Carotenoids (%)	2.65
Tocopherols (%)	0.43
Ascorbic acid (%)	1.25
Phycocyanins (%)	13.51
GSH (mM)	0.245
Total flavonoids (%)	0.87
Total Phenolics (%)	1.73
Proteins %	35.41
Carbohydrates %	22.57
Oils %	16.32

[a]The values are average of triplicate determinations, for alga harvested culture.

Table II
Mineral composition of Spirulina platensis *biomass grown in a culture contain high salt and low nitrogen concentration*

Elements	*Content*[a]
Phosphorus mg/100g	340.63
Calcium mg/100g	0.45
Iron mg/100g	787.00
Selenium mg/100g	1.20
Potassium mg/100g	0.28
Mg mg/100g	0.74
Cr mg/100g	13.70
Cu mg/100g	57.10
Mn mg/100g	23.90
Zn mg/100g	30.00

[a]The values are average of triplicate determinations, for alga harvested culture.

µg/100g, 13.7 µg/100g, 57.1 µg/100g, 787 µg/100g, 23.9 µg/100g, 1.2 µg/100g and 30.0 µg/100g, respectively. According to Michalak and Chojnacka[28] the values of these elements, could be used as a food supplement to help meeting the recommended daily intake of some macron [Na, K, Ca Mg ranging from 8.1 to 17. 9 mg/100g] and micro [Fe, Zn, Mn, Cu ranged from 5.1 to 15.2 mg/100 g] elements.

Fatty acid composition of S. maxima *oil*

In this study, *S. platensis* have a high lipid content (20.40%) compared with other earth vegetables such as soy and sunflower seeds[29,30], thus can be considered as a good source of nutritional energy. It is worth mentioning that the lipid fraction contained high levels of essential polyunsaturated fatty acids compared with traditional vegetables. As shown in table III, the most abundant fatty acids in *S. platensis* were C16:0 (17.13%) and C18:1 ω-7 (22.29%). Also, *S. platensis* contained high levels of essential fatty acids: C18:2 ω-6 (linoleic acid, 10.51%) and ω-6 C18:3 ω-3 (linolenic acid, 10.92%). It is worth noting that the presence of high levels of omega fatty acids, that have potential application in health promotion; prevention from atherosclerosis, protection against arrhythmias, reduce blood pressure, beneficial for diabetic patients, prevent various cancers, anti-inflammatory and immune-regulatory effects and other physiological functions[31].

Table III
Fatty acid composition of Spirulina platensis *grown in a culture contain high salt and low nitrogen concentration*

Fatty acids[a]	*Relative content (%)*[b]
$C_{10:0}$	6.93
$C_{12:0}$	4.84
$C_{14:0}$	4.08
$C_{14:1\ (7),\ n\text{-}7\ (\omega\ 7)}$	8.58
$C_{16:0}$	17.13
$C_{16:1\ (9),\ n\text{-}7\ (\omega\ 7)}$	2.91
$C_{16:2\ (7,10),\ n\ \text{-}7\ (\omega\ 6)}$	4.25
$C_{18:0}$	3.67
$C_{18:1\ (7),\ n\text{-}11\ (\omega\ 11)}$	22.29
$C_{18:1\ (11),\ n\ \text{-}7\ (\omega\ 7)}$	3.84
$C_{18:2\ (9,12),\ n\text{-}6\ (\omega\ 6)}$	10.51
$C_{18:3\ (9,12,15)\ ,\ n\text{-}3\ (\omega\ 3)}$	10.92

[a]Fatty acid was identified based on the retention time of standard fatty acids and MS spectrum of NIST 10.
[b]The relative % of the fatty acid was evaluated through the peak area.

Sensory evaluation of functional biscuits supplemented (FFP) with different level of Spirulina platensis *biomass and phycocyanin*

In the light of the above results, *Spirulina* (S) powder or phycocyanin extracts as an ingredient was supplemented some food products in order to enhance the biofunctional and nutritional quality of their products and improve its stability against auto-oxidative. Different levels of *Spirulina* powder (0.3, 0.6 and 0.9 g/100 g dw) or phycocyanin extracts (3.0%; w/w) were substituted to obtain algae-incorporated biscuits and biscuits without algae were used as control. Sensory evaluation of FFP is an important step to consider the possibility towards an industrial and commercial approach. The main sensory characteristics (colour, odour/aroma, flavour, texture and the global appreciation) of biscuits prepared with different quantity of algae extracts were evaluated by untrained panels (Table IV, V and Fig. 1). In general, the panels showed no preference for the control biscuits than treated samples; however they have no positively correlation of the biscuits with microalgae incorporation

Table IV
Sensory evaluation of functional biscuits supplemented with different levels of Spirulina platensis *biomass*

Sensory characteristics	*Control without algal Cells*	Spirulina platensis *cells levels % d.w*		
		0.3	*0.6*	*0.9*
Colour	24±1.38	23±1.25	24±1.25	24±1.25
Aroma	24±1.25	24±1.25	24±1.25	24±1.25
Flavor	24±1.25	24±1.25	24±1.25	24±1.25
Crispiness	24±1.44	24±1.25	24±1.25	24±1.25
After taste	24±1.25	24±1.25	24±1.25	24±1.25
Overall acceptability	24±1.25	24±1.25	24±1.25	24±1.25

*Mean values in the same row which are not followed by the same letter are significantly different ($p<0.05$). Mean ± standard deviation (n=20)

Table V
Sensory evaluation of functional biscuits supplemented with different levels of phycocyanin

Sensory characteristics	*Control without algal extract*	*Phycocyanin functional biscuits 2 % d. w*
Color	22±0.38	24±1.25
Aroma	22±1.25	23±1.65
Flavor	24±1.25	22±1.66
Crispiness	21±1.44	24±1.35
After taste	21±1.45	22±1.25
Overall acceptability	21±1.46	24±0.98

*Mean values in the same row which are not followed by the same letter are significantly different ($p<0.05$). Mean ± standard deviation (n=20)

(0.3 g/100 g *S. platensis* microalgal biomass addition) compared with control biscuits. The biscuit prepared with the maximum incorporation (0.9 g/100 g DW) had the same appreciated, in terms of colored, odour and texture. In concept of texture, the firmness of the biscuits increased linearly and significantly with increased the amounts of microalgal biomass. These results indicate the positive effect of the algae biomass in the biscuit structure protection. This can be related to the fact that *Spirulina* algae biomass has high proteins and carbohydrates contents (20.4 and 40.57%). Similar observation was reported in literatures, where the microalgae protein and carbohydrate molecules can play an important role on the water absorption process, which promotes the increase of biscuits firmness[32,33].

In terms of colour and tasters, no differentiations were found among all biscuits prepared with different amounts of *S. platensis*. In biscuits prepared with higher algae concentration, the panel did not identify fishy taste that leads to a good global appreciation[34]. These results revealed that biscuits, are traditional and nutritious food, can be healthy and very attractive when prepared with the addition of *Spirulina* (rich in proteins, antioxidants compounds and phycocyanin) biomass or phycocyanin blue pigments. Moreover, the enhancement of textural properties, the high stability of colour and texture and the good nutritional profile of the biscuits obtained, reveal a new niche food market.

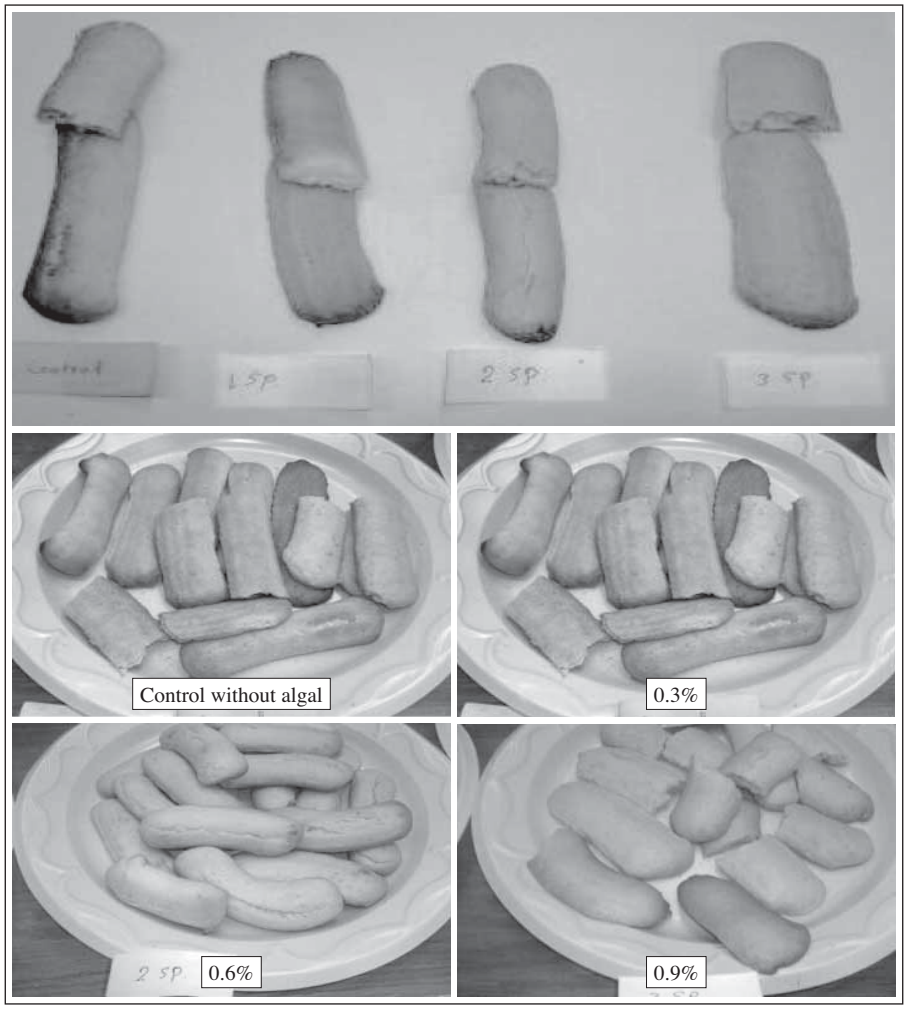

Fig. 1.—Biscuits supplemented with different levels of Spirulina platensis *biomass.*

Table VI
Antioxidant activity (IC_{50} µg/g Scavenging DPPH radical) of functional biscuits supplemented with different levels of Spirulina platensis *biomass*

Storage time (days)	*Control without algal Cells IC_{50} µg/g*	*IC_{50} µg/g Spirulina platensis cells levels %*		
		0.3	*0.6*	*0.9*
Zero time	320.51	80.11	71.22	55.98
5	340.11	84.23	81.21	57.94
10	356.35	89.54	85.23	64.43
15	399.91	92.55	88.11	72.11
20	422.56	120.11	93.33	85.53
25	456.23	134.87	102.55	95.76
30	475.89	155.87	114.44	100.41

Antioxidant activity of functional biscuits during storage

Biscuits produced following the formulation given in table VI and VII. Control biscuits and those produced after supplemented with *S. platensis* extract 0.3, 0.6 and 0.9% and phycocyanin were tested for their radical scavenging using the DPPH radicals. The results of biscuits supplemented with low and high levels of *S. platensis* (0.3% and 0.9% sample (in parenthesis), expressed as IC_{50} values (µg/g), after the production the cooks 0, 5, 10 15, 20 25 and 30 days were 80.11 (55.98), 84.23, 89.54 (57.94), 92.55 (72.11), 120.11 (85.53), 134.87 (95.76) and 155.87 µg/g (100.41 µg/g), respectively (Table VI and VII). Thus, obtained values depending on the levels of supplemented algae levels and increased dose showed a depending manner, and significant differences (<0.05) between IC_{50} values at all levels *S. platensis* was recorded. Increasing antioxidant activity of supplement biscuits could be due to the significant higher phycocyanin content in composition. Similar results were obtained by using phycocyanin in biscuits produced where values IC_{50} were increased by increasing storage periods. But, their values were at all time significantly lowers than that in control samples. IC_{50} values are significantly lower (P<0.05) than those control biscuit samples during storage periods when PC was added a supplemented food ingredients. According to the IC_{50} values of biscuits supplemented with *S. platensis* and phycocyanin, the less the differences in IC_{50} values of scavenged radicals were expected. This antioxidant activity could be due to presence of phycocyanin in *S. platensis*. It is clearly indicated that phycocyanin possesses an antioxidant activity[35,36]. However, significant differences (P<0.05) between IC_{50} values of biscuit supplemented with algae extracted and controls biscuits existed during the 30 days of storage period. After tone week of storage, IC_{50} values of supplemented biscuit tented to be highly decreased from week to week until the end of storage period. Therefore, supplemented of biscuit with algae extract could be recommended due to their antioxidant properties during the storage period.

Table VII
Antioxidant activity (Scavenging DPPH radical IC_{50} µg/g) of functional biscuits supplemented phycocyanin as affected by storage time

Storage time (days)	*Control without phycocyanin IC_{50} µg/g*	*Phycocyanin functional biscuits IC_{50} µg/g*
Zero time	320.51	53.43
5	340.11	55.21
10	356.35	57.45
15	399.91	63.67
20	422.56	78.65
25	456.23	103.15
30	475.89	125.43

The antioxidative effect of algae extracts may have contributed to the oxidative stability of biscuit in addition to natural antioxidants. When added into the biscuit, antioxidants prevent the lipid peroxides formed during storage and delayed oxidation. This could be due to the slow permeation rate of antioxidant components into lipid bi- layer of the biscuits. The data of DPPH for all biscuit samples were considered no rancid and still acceptable until the end of storage periods (30 days). Control exhibited the highest IC_{50} throughout the storage period, showing a high oxidation process. A lower IC_{50} DPPH was observed for all the treated samples, revealing the effectiveness of *S. platensis* extracts of the stabilization of biscuits during storage in room temperatures (30ºC ±3). In manufacturer of bakery products a wide range of fats

and oils ingredients, oils offer the highest potential risk of rancidity due to the autoxidation of unsaturated fatty acids, which are oxidised to hydroperoxides, and subsequently decompose to saturated and unsaturated aldehyde and ketone products[37]. Therefore, the most important changes in food production and storage are the oxidized lipid products[38]. This generally involves the degradation of polyunsaturated fatty acids and the production of secondary decomposition products including carbonyls and hydrocarbon compounds[39] and leading to the development of off-flavours or off- odours[40].

Lipid oxidation product (TBA test) of functional biscuits during storage

Thiobarbituric acid (TBA) values for control and treated biscuit were recorded until day 30. The results showed that TBA value and red color intensity for control samples was much higher than other samples. TBA values of biscuits mixed that had been treated with *Spirulina platensis* extracts were much lower than the control thereby indicating protection to the biscuits against autoxidation during storage periods (Table VIII and IX). In control sample and treated samples (in parenthesis) the TBA values (mM) were 1.02 (0.0), 1.52 (0.2), 2.43 (0.04), 4.32 (0.18), 5.33 (0.24) and 6.36 (0.34) at 5, 10, 15, 20 25 and 30 days, respectively. Therefore, the TBA value for control sample showed the much high values compared to treated samples. All treated samples resulted in significantly lower ($p< 0.05$) TBA values when compared to the control, which indicates that the phycocyanin extracts incorporated into biscuit exhibited antioxidant properties. Several studies showed that a positive relationship between phycocyanin content in *Spirulina* and antioxidant activity (oxidative stability) in certain microalgae products[4,41]. Besides, *S. platensis* had heterogeneous group of molecules and also several vitamins provide strong antioxidant activity[4]. These antioxidant vitamins compounds which are responsible for antioxidant activity in seaweed include vitamin E (α-tocopherol), carotenoids (β-carotene) and vitamin C (ascorbic acid), are responsible for preventing or retarding free radical-induced diseases such as cardiovascular diseases and certain types of cancers[2,13,42,43]. Thus, *S. platensis* may be used as the first line of therapeutic defense against cancer before cancer treatment.

Table VIII
Response of Spirulina platensis *cells supplemented biscuits on lipid oxidation biscuits during storage*

Storage time (days)	*Control without algae*	*TBARs (mM/g)* Spirulina platensis *cells levels %*		
		0.3	*0.6*	*0.9*
Zero time	0.0	0.0	0.0	0.0
5	1.02	0.0	0.0	0.0
10	1.52	0.2	0.0	0.0
15	2.43	0.04	0.02	0.01
20	4.32	0.18	0.05	0.03
25	5.33	0.24	0.07	0.04
30	6.36	0.34	0.13	0.08

Table IX
Response of phycocyanin supplemented biscuits on lipid oxidation biscuits during storage

Storage time (days)	*Control without phycocyanin*	*TBARs (mM/g) Phycocyanin functional biscuits*
Zero time	0.0	0.0
5	1.02	0.0
10	1.52	0.0
15	2.43	0.04
20	4.32	0.28
25	5.33	0.34
30	6.36	0.44

The aforementioned results showed that *S. platensis* is promising sources for new food and functional food products, and can be used to enhance the nutritional value of foods, due to their well-balanced chemical composition. The high quantity of phycocyanin in *S. platensis* may be responsible for strong antioxidant activity. *S. platensis* phycocyanin may act in a similar fashion as reductions by donating the electrons and reacting with free radicals to convert them to more stable product and terminate free radical chain reaction[4,44,45,46,47]. This can indicate that marked antioxidant activity of *S. platensis* cells and its phycocyanin in biscuit seem to be the result of their radical scavenging activity and reducing power. Finally, with the increased demand for health food products in Egypt and consumes microalgae food could be acceptance, the research and development of microalgae as healthy foods will speed up *Spirulina* and other edible microalgae will play an important role in the health and nutrition of human beings.

Conflict of interest

There are no conflict of interest.

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