Functional biscuits with PUFA-ω3 from *Isochrysis galbana*

Luísa Gouveia,[1]* Carla Coutinho,[2] Emanuel Mendonça,[2] Ana Paula Batista,[3] Isabel Sousa,[4] Narcisa M Bandarra[5] and Anabela Raymundo[3]

[1]*Instituto Nacional de Engenharia e Tecnologia Industrial – INETI-DER – UB, Estrada do Paço do Lumiar, Edifício G, 1649-038 Lisbon, Portugal*

[2]*Extensão da Escola Superior de Biotecnologia em Caldas da Rainha, Universidade Católica Portuguesa, Rua Mestre Mateus Fernandes, 2500-237 Caldas da Rainha, Portugal*

[3]*Centro de Investigação de Engenharia Alimentar e Biotecnologia, Instituto Piaget – ISEIT de Almada, Quinta da Arreinela de Cima, 2800-305 Almada, Portugal*

[4]*Technical University of Lisbon, Secção de Ciência e Tecnologia dos Alimentos/DAIAT – Instituto Superior de Agronomia, Tapada da Ajuda, 1349-017 Lisbon, Portugal*

[5]*Departamento de Inovação Tecnológica e Valorização dos Produtos da Pesca, Instituto de Investigação das Pescas e do Mar – IPIMAR, Av. Brasília, 1449-006, Lisbon, Portugal*

Abstract

BACKGROUND: Sweet biscuits, a traditional and nutritious food, can be healthy and very attractive when redesigned to be prepared with the addition of a natural product, the microalgal biomass of *Isochrysis galbana*. This marine microalga is recognised as a rich source of polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic acid (EPA; 20:5ω3), and is a promising ingredient in the food and feed industries. The importance of PUFA-ω3 (an alternative to fish oils) in food, and the need to increase the daily intake of these substances to promote a healthier lifestyle is now well known.

RESULTS: Traditional butter biscuits were enriched with *I. galbana* biomass (1% and 3%) and evaluated in terms of colour, texture and fatty acid profile, within 3 months of storage. *I. galbana* biscuits presented total levels of 100 mg 100 g^{-1} and 320 mg 100 g^{-1} of PUFA-ω3 (EPA + DPA (docosapentaenoic acid; 22:5ω3) + DHA (docosahexaenoic acid; 22:6ω3) for 1% and 3% *I. galbana*, respectively.

CONCLUSION: The enhancement of texture properties, the high stability of colour and texture and the good profile of polyunsaturated fatty acids, with emphasis on EPA and DHA, of the biscuits obtained, reveal a new food market niche opportunity.

Keywords: *Isochrysis galbana*; microalga; biscuits; colour; texture; polyunsaturated fatty acids

INTRODUCTION

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

The importance of ω3 polyunsaturated fatty acids (PUFAs) has been recognised in recent years due to their therapeutic value, with beneficial effects upon human health. These compounds are important building blocks in neonatal retinal and brain development[1,2] as well as being important mainly in the prophylaxis and therapy of chronic and degenerative diseases (reduction of blood cholesterol,[3] protection against cardiovascular, coronary heart diseases, atherosclerosis, diabetes, hypertension, rheumatism, skin diseases, digestive and metabolic diseases as well as cancer).[4–7]

Usually, these compounds are provided by fish and fish oils but global fish stocks are declining due to general over-fishing and overly efficient fishing methods, and the oils derived from fish are sometimes contaminated with a range of pollutants, heavy metals and toxins.[8] Alternative sources of PUFAs are clearly desirable, and some microalgae which synthesise these fatty acids are particularly attractive, especially because these ingredients are naturally encapsulated, retarding oxidation processes.

Microalgal biotechnology is similar to conventional agriculture, but microalgae is a crop of the future

* Correspondence to: Luísa Gouveia, Instituto Nacional de Engenharia e Tecnologia Industrial – INETI-DER – UB, Estrada do Paço do Lumiar, Edifício G, 1649-038 Lisbon, Portugal
E-mail: luísa.gouveia@ineti.pt
Contract/grant sponsor: XXXXX; contract/grant number: 22-05-01-FDR-0007
Contract/grant sponsor: XXXXX; contract/grant number: POCI/EME/58807/2004
Contract/grant sponsor: XXXXX; contract/grant number: PTDC/AGR-ALI/65926/2006
(*Received 7 May 2007; revised version received 12 October 2007; accepted 15 October 2007*)
DOI: 10.1002/jsfa.3166

and has received considerable attention over the last decades, because (1) algae have efficient photosynthetic machinery (when compared with plants and crops); (2) photosynthesis can be extended to areas and climates unsuitable for agricultural activities (including deserts and seashore areas); (3) less or no seasonality is required; (4) algae can act as a medium- or long-term carbon dioxide sink and can diminish the CO_2-induced greenhouse effect; (5) it is a closed life-support system; and (6) the technology involves aquaculture and can be used for wastewater treatment and/or a great variety of biomolecules of commercial interest.

The incorporation of *Isochrysis galbana* microalga biomass can provide an important polyunsaturated fatty acid supplementation[9,10] and other functional important biomolecules, such as pigments,[11] sterols,[12] tocopherols,[13] pharmaceuticals,[14] fibres and oligoelements.

One of the main issues regarding the application of functional ingredients in novel food products is their stability and resistance to severe processing conditions (e.g. high temperatures in biscuit production). The aim of this work was to use *Isochrysis galbana* microalga biomass as a new functional ingredient in short-dough biscuits. Hue and colour intensity (CIELAB system) and texture parameters of the biscuits were determined as a function of pigment concentration over time. The development of a fatty acids profile was followed to evaluate the nutritional value of the biscuits.

EXPERIMENTAL

Microalga

The microalga *Isochrysis galbana* used in this study was obtained from the Mary Park collection and maintained by IPIMAR since 1973. The microalga was grown in 100 L amounts in plastic airlift bioreactors with bubbling air in enriched seawater with Miquel's Medium and Wallerstein's Medium in the ratio 3:1 with $NaNO_3$ and KNO_3 used as the nitrogen source and NaH_2PO_4 and K_2PO_4 as the phosphorus supply. All solutions were autoclaved at 121 °C for 20 min. Solutions of vitamins B_1 and B_{12} were sterilised through a 0.22 μm filter. The culture was kept illuminated with fluorescent lamps (Philips TLM 40W/54RS) at an irradiance level of 196 $\mu mol\, m^{-2}\, s^{-1}$ with a photoperiod of 24:0 h (L:D). The culture was continuously stirred by filtered air and all cultures were kept at 18 ± 1 °C under continuously controlled conditions. The culture salinity was 25‰. The microalga biomass was recovered in the stationary growth phase without flocculation by simply stopping agitation, concentrated by centrifugation, and freeze drying.

Biscuits

The biscuits were prepared using 46.5% flour, 23% sugar, 20% butter, 10% water and 0.5% of baking powder. *I. galbana* biomass was added at 1.0% and 3.0% concentrations (w/w), and a control biscuit with no algal addition was prepared. The biscuits were baked in an oven (Freibol, FB Model) at 180 °C for 30 min. After cooling, biscuits were kept inside plastic bags, in sealed glass jars, at room temperature and protected from light.

Colour

The biscuit colour was measured instrumentally using a Minolta CR-300 (Japan) tri-stimulus colorimeter which was calibrated using a white standard porcelain plate (L^*, 97.46; a^*, −0.02; b^*, 1.72).

The results were expressed in accordance with the CIELAB uniform colour system with reference to standard illuminant D65 (average daylight conditions) and a visual angle of 2°. The parameters determined were L^* which accounts for the lightness (0 for black and 100 white), a^* greeness/redness ($-a^*$ is green and $+a^*$ is red), b^* blueness/yellowness ($-b^*$ is blue and $+b^*$ is yellow). The colour changes during storage are expressed as ΔE^* with the colour of the fresh biscuits (time zero) measured after 1 day of storage as a reference sample. ΔE^* is the total colour difference calculated from the equation

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \qquad (1)$$

The measurements were conducted at 20 °C under the same light conditions (50 mm^2 measuring area per measurement), and replicated five times, in duplicate, after preparation, weekly (during the first month) and after 2 and 3 months of storage under the conditions described above.

Texture

The biscuit texture was measured objectively using a texturometer TA.XT2 (Stable MicroSystems, Surrey, UK) in penetration mode with a cylinder of 2 mm diameter probe plunged 4 mm at 1 mm s^{-1}. The resistance to penetration was measured by the maximum force (in newtons) from the peak on the texturogram which corresponds to the firmness value. Measurements were done in duplicate, five replicates, after preparation, weekly (during the first month) and after 2 and 3 months, as already mentioned.

Fatty acids

The fatty acid profile of *I. galbana* and the biscuit lipid fraction (after preparation and monthly until the third month) was evaluated. Fatty acid methyl esters were prepared according to the method of Lepage and Roy,[15] as modified by Cohen *et al.*[16] Preparation of fatty acid methyl esters was carried out using 25 g of biscuit and 5 mL of the acetylchloride:methanol mixture (1:19 v/v). The esterification was done at 80 °C for 1 h. After cooling, 1 mL of water and 2 mL of *n*-heptane were added to the mixture, which was stirred and centrifuged at 2000 × *g* for 5 min. The organic phase was collected, filtered and dried with anhydrous sodium sulfate. The solvent was removed under nitrogen and the methyl esters solubilised with

n-heptane to a concentration of 100 mg mL^{-1}. Fatty acid methyl esters were separated and quantified using a CP-3800 GC (Varian, Walnut Creek, CA, USA) equipped with 30 m DB-WAX (J&W, Agilent) capillary column (0.25 mm internal diameter, and 0.25 µm film thickness). Injector (split 1:100) and detector (flame ionisation) temperatures were kept constant at 250 °C. The oven temperature program started at 180 °C for 5 min, increased at 4 °C min^{-1} until 220 °C, and kept constant at this temperature for 25 min. The velocity of the carrier gas, He, was kept constant at 1 mL min^{-1}. Quantification was done by multiplication of the relative area of each fatty acid by a corrective factor of 0.956 (based on lipid classes distribution determined by HPLC[17]) and a total lipid content of 20% (biscuit total lipid content) following the calculation proposed by Exler *et al.*[18] Fatty acid methyl esters were identified by comparison with the retention time of individual standards (Sigma, St Louis, USA). All the analytical determinations were done in duplicate, after preparation and 3 months of storage.

Statistics

Data are presented as mean ± standard deviation and subjected to ANOVA–post hoc comparisons–Scheffé test, at the 0.05 probability level, using the StatSoft STATISTICA program, version 6.0.

RESULTS AND DISCUSSION

The biscuits prepared with the *I. galbana* microalga biomass (and control) had an appealing appearance with attractive and innovative hues, as shown in Fig. 1. The odour/aroma of the biscuits was not negatively affected by the incorporation of the microalgal biomass, as reported by sensory evaluation performed by untrained individuals for all characteristics except taste.

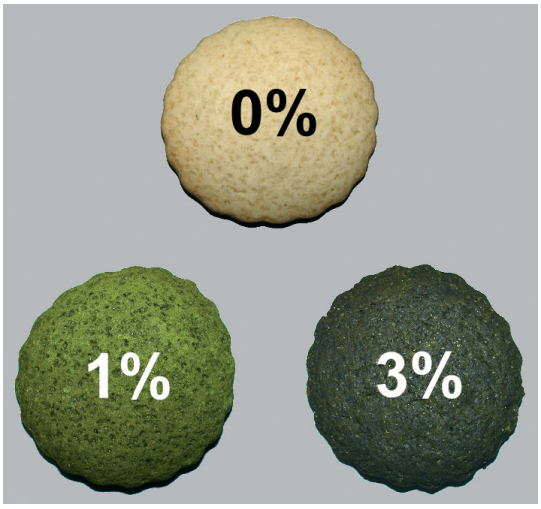

Figure 1. Biscuits with different incorporation levels of *Isochrysis galbana* biomass.

Colour analysis

Effect of microalgal biomass addition

Lightness parameter L^* (Fig. 2a) significantly ($P < 0.05$) decreased with microalgal concentration, which means that the addition of the microalga biomass resulted in darker biscuits, as previously observed in similar biscuits with the addition of *Chlorella vulgaris* microalga biomass.[19]

The evolution of the biscuits' chromaticity coordinates parameters, a^* and b^*, can be observed in Fig. 2b) and 2c), respectively. The biscuits presented quite different ($P < 0.05$) a^* and b^* values, reflecting different colourations. The control biscuit (without microalga) presented a dominant yellow chromaticity (positive b^*) with only a very slight contribution from the a^* parameter ($a^* < 0.2$). The incorporation of microalgal biomass results in significantly ($P < 0.05$) different colours: 1% *I. galbana* biscuit showed a green

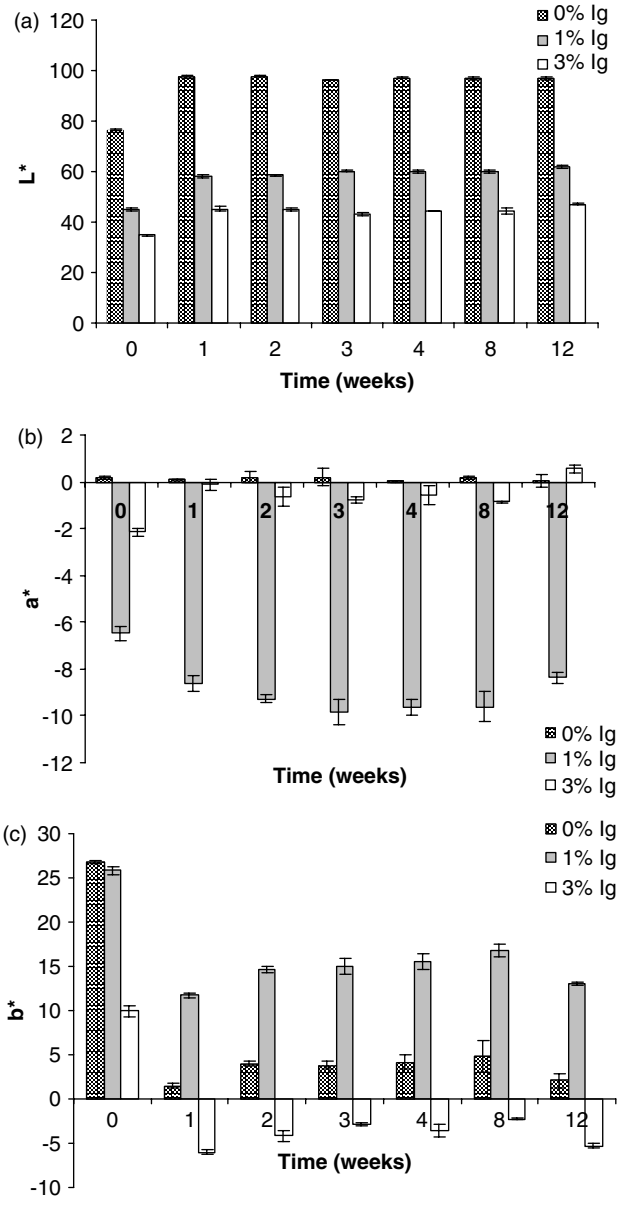

Figure 2. Evolution of colour parameters for the biscuits, L^*, a^* and b^* (*a*, *b*, *c*, respectively), with 0%, 1% and 3% *Isochrysis galbana* biomass incorporation, within 3 months of storage.

chromaticity (negative a^*) with positive b^* values (yellow domain); while 3% *I. galbana* biscuit presented a small and negative a^* (green) and b^* (blue) values.

Since the three biscuits (0%, 1%, 3% *I. galbana*) are positioned on very distant points on the CIELAB colour space, it can be easier to compare the samples by calculating the chroma - saturation ($C^*_{ab} = (a^{*^2} + b^{*^2})^{1/2}$) and hue angle ($h_{ab} = \arctan(b^*/a^*)$), rather than a^* and b^* separately. The control biscuit (0% *I. galbana*) presented an almost pure yellow hue ($h_{ab} = 87–90°$), although the saturation is very low ($C^*_{ab} = 2–5$), resulting in a dull coloration (Fig. 1). With the addition of 1% *I. galbana*, the biscuit hue turned greener, with h_{ab} values varying from 120 to 126°, and chroma increased ($C^*_{ab} = 15–19$). However, for higher biomass concentrations (3% *I. galbana*) the biscuit colour turned browner ($h_{ab} = 250–270°$; $C^*_{ab} = 2–6$), similar to the colour of *Isochrysis* dry biomass.

Effect of storage time

Biscuit coloration underwent significant ($P < 0.05$) changes during the first week after preparation, with total colour differences (ΔE^*) around 30 for 0% *I. galbana*, 19 and 17 for 1% and 3% *I. galbana*, respectively (Fig. 3). Consequently, the colour of the biscuit with microalga addition is more stable than the colour of the control biscuit, as previously reported by Gouveia *et al*.[19]

The main colour changes observed at the beginning of the storage time can be associated with a fast degradation of the microalga pigments, during the first days after preparation, resulting essentially from oxidation processes. After this period, a stabilisation ($P > 0.05$) of the colour parameters was achieved, with $\Delta E^* < 5$, which is the value required to discern the samples visually.[20] This behaviour can be an important issue for commercial purposes.

Texture analysis

The firmness of the biscuits increased linearly and significantly ($P < 0.05$) with microalgal biomass, as

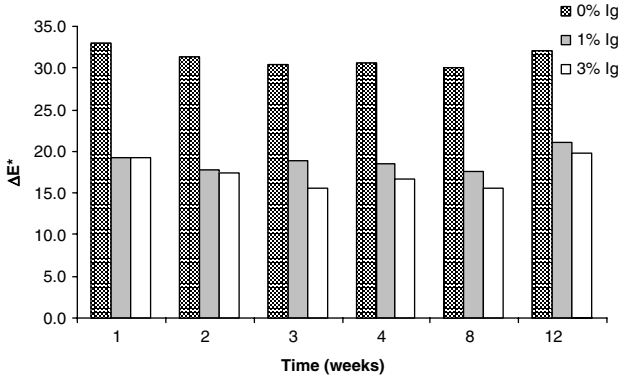

Figure 3. Total colour difference ($\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$) of the biscuits with 0%, 1% and 3% *Isochrysis galbana* biomass incorporation, within 3 months storage.

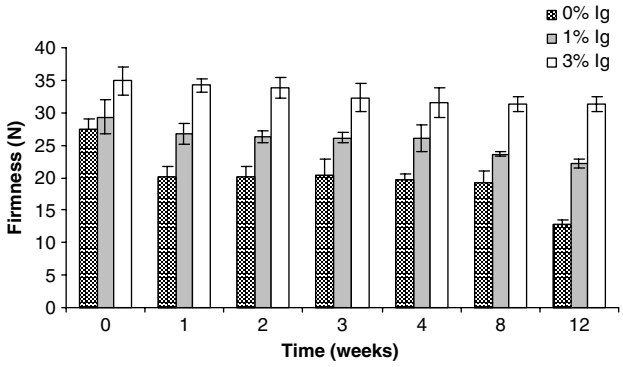

Figure 4. Evolution of firmness values for the biscuits, with 0%, 1% and 3% *Isochrysis galbana* biomass incorporation, within 3 months storage.

can be seen in Fig. 4. These results are in agreement with previous studies with similar materials.[19]

According to the American Association of Cereal Chemists[21] a biscuit is a solid emulsion of sucrose, lipids and non-gelatinised starch. This morphology is responsible for the biscuit structure and texture. The main factors affecting these properties is moisture content and water mobility, which are greatly affected by the interaction with hydroxyl groups present in the matrix.[21,22]

The replacement of a small amount of flour by microalgae biomass, resulted in the inclusion of a complex biomaterial, rich in proteins (~35%) and polysaccharides (~10%).[10] These molecules have an important role on the water absorption process, which promote the increase of biscuits firmness, resulting in more compact structure, as observed by Piteira.[23]

The texture of the *I. galbana* biscuits remained very stable ($P > 0.05$), especially the 3% *I. galbana*; while the control biscuit firmness decreased significantly ($P < 0.05$) during the first and 12th weeks of the study. The observed beneficial effect of micralgal incorporation on the stability of the biscuit texture associated with the previous colour stability results lead us to think that the shelf life of this type of product can be enhanced by the incorporation of microalgae, with a positive commercial impact.

Fatty acids profile

The fatty acid profile of *I. galbana* showed the presence of polyunsaturated fatty acids as a dominant group, followed by saturated and monounsaturated fatty acids (40%, 30% and 19%, respectively), with EPA as the most abundant (Fig. 5).

The main saturated fatty acids present are miristic acid (14:0) and palmitic acid (16:0) (49% and 41%, respectively). The palmitoleic acid (16:1ω7) is the relevant monounsaturated fatty acid, corresponding to 77% of this fraction. The major polyunsaturated fatty acids present are EPA at a high level (55%), followed by DHA and α-linolenic acid (18:3ω3) (13% and 5%, respectively). These results are in agreement with those reported in other studies.[12,24,25]

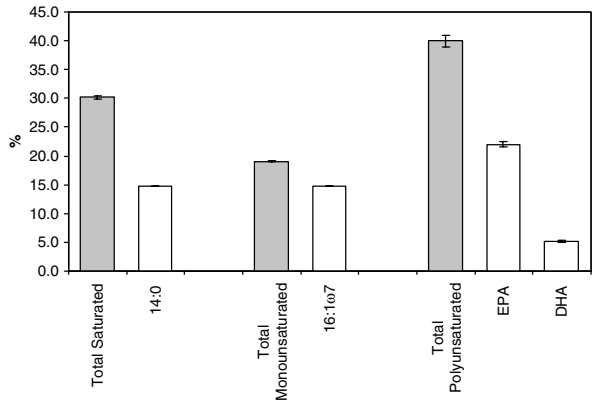

Figure 5. Total and main saturated, mono-unsaturated and polyunsaturated fatty acids in *Isochrysis galbana* biomass.

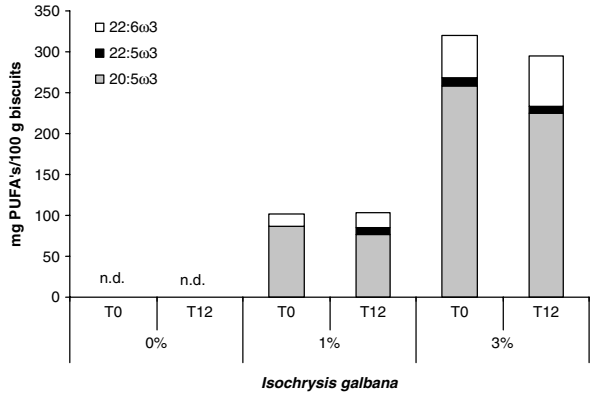

Figure 6. Evolution of ω3-polyunsaturated fatty acids, of biscuits with 0%, 1% and 3% *Isochrysis galbana* biomass incorporation, at 0 and 12 weeks. EPA, eicosapentaenoic acid (20:5ω3); DPA, docosapentaenoic acid (22:5ω3); and DHA, docosahexaenoic acid (22:6ω3).

The fatty acids profile of the biscuits is clearly related to butter,[26] with a 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 correspond to 6–9% (4–5% linoleic acid; 18:2ω6), the highest levels being for 3% *I. galbana* biscuits (55% linoleic acid, 15% EPA, 6% α-linolenic acid and 3% DHA) (Table 1).

On biscuits produced with *Isochrysis* microalga the ω3-fatty acids (DHA, EPA and DPA) from the microalga remained present after the thermal processing of baking. This can be seen in Table 1 and Fig 6. whereas all the other fatty acids, mainly provided by butter, showed large variations. The authors suggest that the microalgae cells could resist thermal treatment, encapsulating the fatty acid molecules, thus protecting them from oxidation.

I. galbana biscuits presented PUFA-ω3 levels (EPA + DPA + DHA) of 100 mg 100 g^{-1} and 320 mg 100 g^{-1} biscuit (Fig. 6), for 1% and 3% microalgal biomass incorporation, respectively. These values reflect an important source of PUFA-ω3 with moderate biscuit consumption, as the recommendations for dietary intake in healthy adults is 500 mg day^{-1}.[27]

The development of the fatty acid profile of the biscuits with time revealed that long-chain PUFAspresented good stability ($P < 0.05$) maintaining a high nutritional level even after 3 months of storage at room temperature and without special carrier conditions (temperature, light and atmosphere). However, a significant ($P < 0.05$) decrease of total saturated fatty acids and an increase on total mono-unsaturated fatty acids was observed, probably due to an interconversion (oxidation) between these compounds, which may be related to colour fading of the biscuits.

CONCLUSIONS

Sweet biscuits, a traditional and nutritious food, can be healthy and very attractive when prepared with the addition of a natural microalgal biomass of

Table 1. Total and main saturated, mono-unsaturated and polyunsaturated fatty acids (mg 100 g^{-1}) in biscuits with *Isochrysis galbana* incorporation

		Isochrysis galbana (w/w)*		
	Acid	0%	1%	3%
14:0	Miristic	2159 ± 12^{a}	2135 ± 7^{a}	2080 ± 14^{b}
16:0	Palmitic	6624 ± 77^{a}	6474 ± 30^{ab}	6314 ± 1^{b}
Total saturated		11737 ± 175^{a}	11482 ± 35^{a}	11492 ± 8^{a}
18:1ω 9	Oleic	4374 ± 45^{a}	4246 ± 6^{a}	4340 ± 411^{a}
Total mono-unsaturated		5819 ± 19^{a}	5640 ± 6^{a}	5633 ± 134^{a}
18:2ω6	Linoleic	877 ± 22^{a}	967 ± 28^{a}	949 ± 39^{a}
18:3ω3	α-Linolenic	73 ± 2^{a}	87 ± 1^{b}	99 ± 5^{b}
20:4ω6	Arachidonic	16 ± 1^{a}	34 ± 1^{a}	38 ± 1^{a}
20:5ω3	EPA**	ND	87 ± 2^{a}	258 ± 33^{b}
22:5ω3	DPA**	ND	ND	10 ± 1
22:6ω3	DHA**	ND	14 ± 1^{a}	52 ± 7^{b}
Total polyunsaturated		1161 ± 73^{a}	1458 ± 39^{ab}	1734 ± 111^{b}

* Mean value ± SD. Different letters in the same row correspond to significant differences ($P < 0.05$).
** EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.
ND, not detected.

Isochrysis galbana (rich in PUFAs, particularly EPA). The enhancement of textural properties, the high stability of colour and texture and the good profile of polyunsaturated fatty acids, with an emphasis on EPA and DHA, of the biscuits obtained, reveals a new niche food market.

ACKNOWLEDGEMENT

The authors would like to acknowledge for financial support of the projects 'Biotechnology of marine organisms' from FEDER, QCAIII (project number 22-05-01-FDR-0007), 'Solar Ponds: Bioconvection Modeling' from FCT (project number POCI/EME/58807/2004) and 'Pigments, antioxidants and PUFAs in microalgae based food products – functional implications' from FCT (project number PTDC/AGR-ALI/65926/2006).

REFERENCES

1 Crawford M, Placental delivery of arachidonic acid and docosahexaenoic acids: implication of the lipid nutrition of preterm infants. *Am J Clin Nutr* **71**:275S–284S (2000).
2 Thies F, Garry JM, Yagoob P, Rerkasem K, Williams J, Shearman CP, *et al.*, Association of *n*-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: a randomized controlled trial. *Lancet* **361**:477–485 (2003).
3 Bønaa KH, Bjerve KS, Straume B, Gram IT and Thelle D, Effect of eicosapentaenoic and docohexaenoic acids on blood pressure in hypertension: a population-based intervention trial from the Tromsø study. *N Engl J Med* **322**:795–801 (1990).
4 Simopoulos AP, Historical perspective, conference conclusions and recommendations, and actions by federal agencies, in *Health Effects of Polyunsaturated Fatty Acids in Seafood*, ed. by Simopoulos AP, Kifer RR and Martin RE. Academic Press, Orlando, pp. 3–29 (1986).
5 Dratz EA and Deese AJ, The role of docosahexaenoic acid (22:63) in biological membranes: examples from photoreceptores and model membrane bilayers, in *Health Effects of Polyunsaturated Fatty Acids in Seafood*, ed. by Simopoulos AP, Kifer RR and Martin RE. Academic Press, Orlando, pp. 319–352 (1986).
6 Vitale JJ, Lipids, host defenses and immune function, in *Dietary Fat Requirements in Health and Development*, ed. by Beare-Rogers J. AOCS, Champaign, pp. 137–141 (1988).
7 Ötles S and Pire R, Fatty acid composition of *Chlorella* and *Spirulina* microalgae species. *J AOAC Int* **84**:1708–1714 (2001).
8 Qi B, Fraser T, Mugford S, Dobson G, Sayanova O, Butler J, *et al.*, Production of very long chain polyunsaturated omega-3 and omega-6 fatty acids in plants. *Nat Biotechnol* **22**:739–745 (2004).
9 Grima EM, Pérez JAS, Camacho FG, Fernández FGA, Alonso DL and Castillo CIS, Preservation of the marine microalga *Isochrysis galbana*: influence on the fatty acid profile. *Aquaculture* **123**:377–385 (1994).
10 Fidalgo JP, Cid ATE, Sukenik A and Herrero C, 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 (1998).
11 Flynn KJ, Zapata M, Garrido JL, Oepik H and Hipkin CR, Changes in carbon and nitrogen physiology during ammonium and nitrate nutrition and nitrogen starvation in *Isochrysis galbana*. *Eur J Phycol* **28**:47–52 (1993).
12 Volkman JK, Smith DJ and Eglinton G, Sterol and fatty acid composition of four marine *Haptophycean* algae. *J Marine Biol Assoc UK* **61**:509–527 (1981).
13 Fábregas J and Herrero C, Vitamin content of four marine microalgae. Potential use as source of vitamins in nutrition. *J Ind Microbiol Biotech* **5**:259–264 (1990).
14 Fábregas J, Garcia D, Fernandez-Alonso M, Rocha AI, Gomez-Puertas P, Escribano JM, *et al.*, *In vitro* inhibition of the replication of haemorrhagic septicaemia virus (VHSV) and African swine fever virus (ASFV) by extracts from marine microalgae. *Antiviral Res* **44**:67–73 (1999).
15 Lepage G and Roy C, Direct transesterification of all classes of lipids in a one step reaction. *J Lipid Res* **27**:114–119 (1986).
16 Cohen Z, Vonshak A and Richmond A, Effect of environmental conditions on fatty acid composition of the red algae *Porphyridium cruentum*: correlation to growth rate. *J Phycol* **24**:328–332 (1988).
17 Bandarra NM, Batista I, Nunes ML and Empis JM, Seasonal variation in the chemical composition of horse-mackerel (*Trachurus trachurus*). *Eur Food Res Technol* **212**:535–539 (2001).
18 Exler J, Kinsella JE and Watt BK, Lipid and fatty acids of important finfish: new data for nutrient tables. *J Am Oil Chem Soc* **52**:154–159 (1975).
19 Gouveia L, Batista AP, Miranda A, Empis J and Raymundo A, *Chlorella vulgaris* biomass used as colouring source in traditional butter cookies. *Innov Food Sci Emerg Technol* **8**:433–436 (2007).
20 Castellar MR, Obon JM and Fernandez-Lopez JA, The isolation and properties of a concentrated red-purple betacyanin food colourant from *Opuntia stricta* fruits. *J Sci Food Agric* **86**:122–128.
21 Hoseney RC, Wade P and Finley JW, Soft wheat products, in *Wheat Chemistry and Technology*, vol. II, 3rd edition, ed. by Pomeranz Y. *American Association of Cereal Chemists Monograph Series*, pp. 407–456 (1988).
22 Cornillon P and Salim L, Characterization of water mobility and distribution in low- and intermediate-moisture food systems. *Magn Reson Imaging* **18**:335–341.
23 Piteira MFC, *Alimentos Funcionais – Incorporação de Fibras em Bolachas*, PhD thesis. Technical University of Lisbon, Agronomy Superior Institute (2006).
24 Liu C and Lin L, Ultrastructural study and lipid formation of *Isochrysis* sp. CCMP1324. *Bot Bull Acad Sinica* **42**:207–214 (2001).
25 Bandarra NM, Pereira PA, Batista I and Vilela MH, Fatty acids, sterols and α-tocopherol in *Isochrysis galbana*. *J Food Lipids* **18**:25–34 (2003).
26 Özkanli O and Kaya A, Storage stability of butter oils from sheep's non-pasteurized and pasteurized milk. *Food Chem* **100**:1026–1031 (2007).
27 International Society for the Study of Fatty Acids and Lipids, *Recommendations for Dietary Intake of Polyunsaturated Fatty Acids in Healthy Adults*. ISSFAL, Devon, UK (2004).