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Bioaccessibility of Carotenoids and Tocopherols in Marine Microalgae, *Nannochloropsis sp.* and *Chaetoceros sp.*

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ABSTRACT

Microalgae can produce various natural products such as pigments, enzymes, unique fatty acids and vitamin that benefit humans. The objective of the study is to study the bioaccessibility of carotenoids (β-carotene and lycopene) and vitamin E (α- and β-tocopherol) of *Nannochloropsis oculata* and *Chaetoceros calcitrans*. Analyses were carried out for both the powdered forms of *N. oculata* and *C. calcitrans*, and the dried extract forms of *N. oculata* and *C. calcitrans*. *In vitro* digestion method together with RP-HPLC was used to determine the bioaccessibility of carotenoids and vitamin E for both forms of microalgae. Powdered form of *N. oculata* had the highest bioaccessibility of β-carotene (28.0 ± 0.6 g kg^{-1}), followed by dried extract *N. oculata* (21.5 ± 1.1 g kg^{-1}), dried extract *C. calcitrans* (16.9 ± 0.1 g kg^{-1}), and powdered *C. calcitrans* (15.6 ± 0.1 g kg^{-1}). For lycopene, dried extract of *N. oculata* had the highest bioaccessibility of lycopene (42.6 ± 1.1 g kg^{-1}), followed by dried extract *C. calcitrans* (41.9 ± 0.6 g kg^{-1}), powdered *C. calcitrans* (39.7 ± 0.1 g kg^{-1}) and powdered *N. oculata* (32.6 ± 0.7 g kg^{-1}). Dried extract *C. calcitrans* had the highest bioaccessibility of α-tocopherol (72.1 ± 1.2 g kg^{-1}). However, β-tocopherol was not detected in both dried extract and powdered form of *C. calcitrans*. In conclusion, all samples in their dried extract forms were found to have significantly higher bioaccessibilities than their powdered forms. This may be due to the disruption of the food matrix contributing to a higher bioaccessibility of nutrients shown by the dried extract forms

INTRODUCTION

The term microalgae refers to the aquatic microscopic plants (organisms with chlorophyll *a* and a thallus not differentiated into roots, stem and leaves), and the oxygenic photosynthetic bacteria, that is, the cyanobacteria, formerly known as Cyanophyceae (Tomaselli, 2004). Microalgae are either unicellular, or are multicellular without highly specialised tissues. They are the primary food source for larvae and juveniles of various aquatic invertebrates and fishes including those species used in mariculture (Brown *et al*., 1997).

Interest in microalgae has greatly increased for different reasons. The potential for microalgae to enhance nutritional content of conventional food preparations is great as microalgae represent a valuable source of nearly all essential vitamins (for example, A, B1, B2, B6, B12, C, E, nicotinate, biotin, folic acid and pantothenic acid) (Beeker, 2004). Microalgae can also be an important source of carotenoids for commercial use,

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since these compounds can be obtained in high yield (Mendes *et al.*, 1995). Besides, microalgae also act as probiotic agents that positively affect the health of humans. Today, microalgae, marketed as health food or food supplements, are commonly sold in the form of tablets, capsules and liquids. They are also incorporated in pastas, snack food, candy bars or gums, in drink mixes and beverages, to name a few, either as a nutritional supplement or as a source of natural colourant.

Bioaccessibility or digestibility refers to the amount of a food component that is released from the food matrix and, for some components, constitutes the maximum amount available for absorption. In addition, fat-soluble components must be incorporated into mixed micelles before absorption. Thus, the efficiency of micellisation (quantities transferred into the aqueous-micellar fraction) is used as an estimate of the relative bioavailability of fat-soluble components, whereas, in combination with the utilisation of the Caco-2 cell line as a surrogate for small intestinal enterocytes, it has been employed as a model for human absorption (Failla & Chitchumroonchokchai, 2005).

The role of carotenoids and tocopherols in the prevention of chronic diseases and their health promoting mechanisms has been well documented (Rao & Rao, 2007; Traber, Frei & Beckman, 2008). Studies had been conducted to determine the amount of carotenoids and tocopherols in different microalgae (Carballo-Cárdenas *et al*., 2003; Brown, McCausland & Kowalski, 1998). However, bioaccessibility of carotenoids and tocopherols from microalgae has scarcely been evaluated in gastrointestinal models. Thus our aim was to assess the bioaccessibility of carotenoids (β-carotene and lycopene) and tocopherols (α, β, δ and γ tocopherol) in powdered and extract forms of two tropical microalgae namely *Nannochloropsis oculata* and *Chaetoceros calcitrans* by using an *in vitro* digestion model.

MATERIALS AND METHODS

Chemicals

All type of solvents of analytical grade and HPLC grade were purchased from Merck (Darmstadt, Germany). All other chemicals of analytical grade and standards were purchased from Sigma (MO, USA) unless otherwise noted.

Microalgae cultures

Two samples of microalgae were used, namely *Nannochloropsis oculata* and *Chaetoceros calcitrans*. The sample of *N. oculata* in seawater was obtained from the photo-bioreactors at the Aquatic Animal Unit of Faculty of Veterinary Medicine, UPM. The samples of *C. calcitrans* were prepared by culturing it using Conway medium (Tompkins *et al*., 1995). After harvesting, the microalgae was sent to the Marine Science and Aquaculture Laboratory of the Bioscience Institute for centrifugation by using HIMAC High Speed Refrigerated Centrifuge Models CR 22GII / CR21GII (Hitachi, Tokyo, Japan). *Nannochloropsis oculata* was centrifuged at 2000 rpm and at a temperature of 25°C. Each round of the centrifugation was for 7 minutes. The *Chaetoceros calcitrans* was centrifuged at 8000 rpm for 15 minutes. Both the resultant cell pellets were re-suspended in 100 mL of distilled water, re-centrifuged and the supernatant again discarded. The washed pellets were frozen overnight at -80°C. Samples were freeze-dried to alleviate the moisture content of the sample. Both dried samples were kept in a refrigerator at 4°C prior to analysis.

Extraction of carotenoids

Carotenoids were extracted from *N. oculata* and *C. calcitran* by adopting a method described by Reboul *et al.* (2006). In brief, 0.5 g of microalgae powder was added to 10 ml of methanol containing 0.57% magnesium

carbonate. Then, samples were homogenated for 30s using a vortex. Then, 10 ml of trichloromethane which contained 0.005% butylated hydroxyl toluene was added. Samples were then homogenised for a further 30s in the vortex blender. Ten millilitres of distilled water were added after a rest of 15 minutes. Samples were centrifuged (2000g for 10 minutes) by using Hettich Universal 32R centrifuge (Hettich, Tuttlingen, Germany)

The lower phases of the samples were collected. The upper phases were repeatedly extracted over three rounds. The extracts of the lower layer were then pooled, evaporated to dryness, re-dissolved in 8 ml of acetonitrile / dichloromethane (50:50; v / v). Then, the extract was filtered with Whatman polytetrafluoroethylene (PTFE) 0.22 μm syringe filter and the filtrate was injected into a HPLC valve with 1 ml syringe. To obtain dried extracts, the dried microalgae extracts after evaporation were dissolved in 5 ml trichloromethane and flushed with nitrogen in a dark room until dried.

Extraction of tocopherols

Tocopherols were extracted from *N. oculata* and *C. calcitran* by adopting a method described by Donato, Vilela & Bandarra (2003). Briefly, 0.5 g of freeze-dried powdered microalgae, 2 ml ethanol and 10 mg ascorbic acid were mixed together. The mixture was swirled for 2 minutes and 3 ml of n-hexane was added. A second swirling of the mixture was then done for 2 minutes, followed by ultrasonic treatment by using 5510 Branson model ultrasonic (Branson Ultrasonic Corp., CT, USA) over a 20-minute period. After this treatment to disrupt the cellular wall, 2 ml of distilled water was added and the mixture was stirred for 1 minute and centrifuged at 2000 g for 10 minutes. The hexane layer (upper phase) was collected. Then, a second and third extraction of the pellets was done, respectively, with 2 ml and 1 ml of n-hexane, but without ultrasonic treatment and water addition. Different organic phases (hexane layers) were pooled and passed through the anhydrous sodium sulphate. The extract was then filtered with Whatman polytetrafluoroethylene (PTFE) 0.22 μm syringe filter and the filtrate was injected into HPLC valve with a 1 ml syringe. To obtain dried extracts, the hexane layers were flushed with nitrogen in a dark room until dried.

In vitro digestion

The amounts of bioaccessible carotenoids and tocopherols were determined according to *in vitro* digestion method by Reboul *et al.* (2006). Briefly, 1.0 g of powdered or dried extract of microalgae was mixed with 32 ml of NaCI 0.9% containing 150 μmol / L β-Hydroxy toluene (BHT). The mixtures were homogenised for 10 minutes at 37°C in a shaking water bath. pH was adjusted to 4 with 1M hydrochloric acid (HCl), then 2 ml of porcine pepsin was added (40 mg mL^{-1} in 0.1 M HCI). The homogenates were incubated at 37°C in a shaking water bath for 30 minutes. The pH of the partially digested mixtures was raised to 6 by adding sodium bicarbonate. After that, a mixture of porcine bile extract and pancreatin were added [9 ml containing 2 mg mL^{-1} pancreatin and 12 mg mL^{-1} bile extract in 100 mmol L^{-1} trisodium citrate (BDH, Poole, England), pH6.0]. Then, 4 ml of porcine bile extract at 0.1 g mL^{-1} was added. Samples were incubated in a shaking water bath at 37°C for 30 minutes to complete the digestion process.

Micellar fraction isolation from digesta

Micelles were separated from oil droplets by ultracentrifugation (44300 rpm for 30 minutes at 4°C) using Beckman Optima XL-100K ultracentrifuge (Beckman, CA, USA). The aqueous fractions were collected from the centrifuge tube. Then, the aqueous fractions were passed through Whatman

filter paper 0.2μm. The aliquots were then stored at -80°C in a freezer until analysis.

Analysis of carotenoids and tocopherols

The micelle fraction aliquots were subjected to the extraction process mentioned above. For carotenoids, after recovery and evaporation of the dichloromethane phase, the volume was adjusted to 1.5 ml with acetonitrile / dichloromethane (50:50; v / v). For tocopherols, the hexane layers were pooled and passed through the anhydrous sodium sulfate. For both carotenoids and tocopherols, the extracts were filtered with Whatman polytetrafluoroethylene (PTFE) 0.22 μm syringe filter and the filtrate was injected into a HPLC valve with 1 ml syringe.

HPLC analyses were performed with the Agilent HPLC Series 1100 (Model G1313A, Agilent Technologies, Germany) equipped with degasser, quaternary pump, auto sampler and diode array detector. Carotenoids were separated by HPLC using a RP-C18 (250nm x 4.6mm, 5 μm) stainless steel column (Zorbax Eclipse model XDB-C18, Agilent Technologies, USA). The mobile phase for β-carotene and lycopene was an isocratic acetonitrile – methanol - dichloromethane (containing 50 mmol mL^{-1} ammonium acetate) (75:20:5 by vol). Carotenoids (β-carotene and lycopene) were detected at 450 nm and identified by retention time and spectral analysis (from 300 to 550nm) in comparison with standards. Injected volume into HPLC was set as 50 μl and the flow rate during separation was set as 1ml min^{-1}. The column temperature was set to 21 °C. The elution time was 45 minutes for a sample and the post time was 5 minutes. β-carotene and lycopene were quantified by using calibration curves prepared with pure standards in the range of 25-250 μg mL^{-1}.

Tocopherols were detected at 295nm and identified by retention time and spectral analysis (from 200 to 400nm) in comparison with standards. The column was a C18-Nucleosil (250nm x 4.6mm, 5 μm) (Macherey-Nagel, Germany), and the mobile phase was n-hexane and isopropanol (99:1 by vol). Injected volume into HPLC was set as 50 μl and the flow rate during separation was set as 1.4 ml min^{-1}. The column temperature was set to 22°C. The elution time was 15 minutes for a sample and post-time was 5 minutes. A mixed standard *of* α-, β-, δ- and γ-tocopherol in the range of 50-250 μg mL^{-1} was used to quantify the four tocopherols.

Data analysis

Computer software statistical package for social sciences version 15.0 (SPSS Inc, IL, USA) was used to analyse the data. One way ANOVA and Bonferroni post-hoc test were applied to see the significant differences ($p<$ 0.05) in bioaccessibility of β-carotene, lycopene and four tocopherols (α-, β-, δ-, and γ-tocopherol) among powdered forms and dried extract forms of two microalgae, namely *N. oculata* and *C. calcitran*.

RESULTS AND DISCUSSION

Bioaccessibility of carotenoids

Figure 1 shows the bioaccessibility of α-carotene in the CP, CE, NP, and NE for absorption after the digestion process. The figure shows that the dried extract form of *N. oculata* (NE) had significantly higher ($p<0.05$) bioaccessibility of β-carotene (28.0 ± 0.6 g kg^{-1}) compared to NP (21.5 ± 1.1 g kg^{-1}), CE (16.9 ± 0.1 g kg^{-1}), and CP (15.6 ± 0.1 g kg^{-1}).

The bioaccessibility of β-carotene in NP and NE were significantly higher ($p<0.05$) than in both forms of *C. calcitrans.* At the same time, the results also showed that both extract forms of the microalgaes were found to have higher bioaccessibility of β-carotene than their powdered forms.

Figure 2 shows the availability of lycopene absorbtion by the gastrointestinal tract after simulated digestion process for CP, CE, NP, and NE. The result showed that

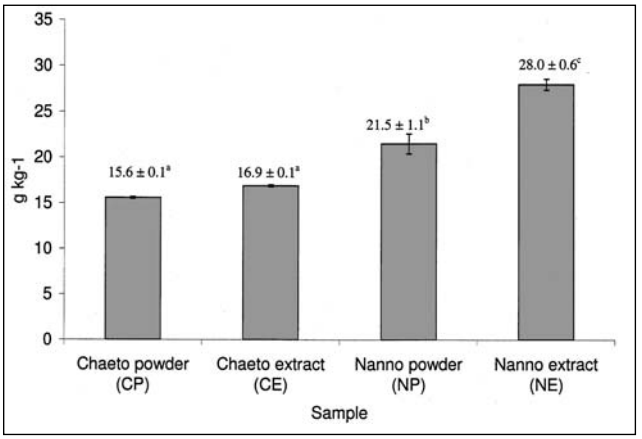

Figure 1. Bioaccessibility of beta-carotene in powdered and dried extract form of *Chaetoceros calcitrans* and *Nannochloropsis oculata* microalgae

Note: Values with different superscripts indicate significant difference at 0.05 level.

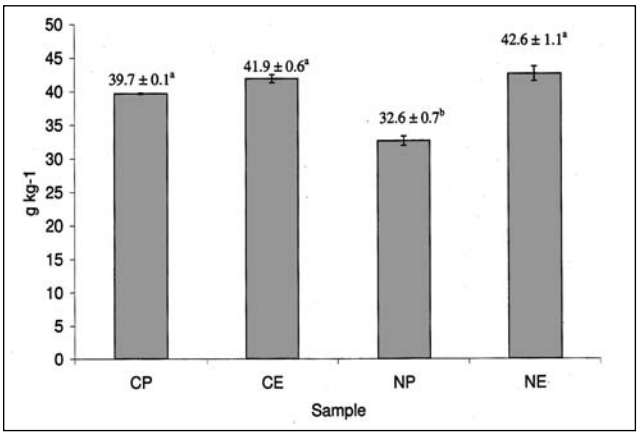

Figure 2. Bioaccessibility of lycopene in powdered and dried extract form of *Chaetoceros calcitrans* and *Nannochloropsis oculata* microalgae

Note: Values with different superscripts indicate significant difference at 0.05 level.

NE has the highest bioaccessibility of lycopene (42.6 ± 1.1 g kg^{-1}), followed by CE (41.9 ± 0.6 g kg^{-1}), and CP (39.7 ± 0.1 g kg^{-1}) respectively. NP has the lowest bioaccessibility of lycopene (32.6 ± 0.7 g kg^{-1}).

One way ANOVA test was carried out to determine the significant differences between the CP, CE, NP, and NE in terms of lycopene's bioaccessibility. The test showed that there were no significant differences among the bioaccessibility of lycopene in CP, CE, and NE. However, bioaccessibility of lycopene in NP was found to be significantly lower than those of CP, CE, and NE. Similarly with β-carotene, both the dried extract forms of the two microalgae were found to be higher compared to their powdered forms.

Previous studies have shown that the bioavailability and bioaccessibility of carotenoids from meals are influenced by a number of factors in addition to the

carotenoid content. These include the manner in which the food was processed and cooked, as well as the content of lipids and fibre in meals (Rock & Swendseid, 1992; Stahl & Sies, 1992; Erdman, Bierer & Gugger, 1993; Shiau *et al*., 1994).

In this study, the bioaccessibility of β-carotene and lycopene in the dried extract form for both *C. calcitrans* and *N. oculata* were found to be greater ($p<0.05$) compared to their powdered forms. It may be due to the dried extracts being more 'disrupted' than the powdered forms. According to Furr & Clark (1997), in order to make carotenoids accessible for absorption, mechanical as well as chemical disruption of the food matrix is important. By mechanical disruption, the surface area for the digestive enzymes to attack enlarges and thereby the carotenoids are more easily released from their matrix. Another study also found that when juices and extracts were assayed, higher amounts of free forms (hydrolysis) were obtained, suggesting that the solubilisation of the substrates (that is, ester forms) from the matrix into the media is a critical factor for efficient hydrolysis (Granado-Lorencio *et al*., 2007)

Other studies also showed increased bioaccessibility and bioavailability after disruption of the food matrix. A seven-fold and an almost five-fold improvement of the β-carotene accessibility were shown after homogenisation of raw and cooked carrot samples (Poor *et al.,* 1993*)*. Van Zeben & Hendriks (1948) showed an almost five-fold increase in concentration of plasma carotenes when women consumed cooked carrots homogenised in a mixer compared with unhomogenised carrots. At the same time, in the study conducted by Toronnen *et al.* (1996), the serum β-carotene response in women was almost twice as high after consumption of carrot juice compared with raw carrots. Gartner, Stahl & Sies (1997) reported the peak lycopene concentration in chylomicrons to be about twice as high after consumption of tomato paste compared with fresh tomatoes.

There was no significant difference ($p>0.05$) found on the bioaccessibilities of α-carotene and lycopene between the extract form and powdered form of *C. calcitrans* (CE and CP). This could be due to the fact that *C. calcitrans* is a diatom. A characteristic feature of diatom cells is that they are encased within a unique cell wall made of silica (hydrated silicon dioxide) called a frustule. Their cell walls are relatively dense and comprise two separate valves or shells. These attributes of the diatom's cell wall may lead to the low digestible level in *C. calcitrans* and may affect the bioaccessibility of nutrients or nutrient absorption.

The bioaccessibility of lycopene in NP was found to be significantly lower ($p<0.05$) compared to NE, CP, and CE (Figure 2). It may be due to the loss of carotenoids in NP. The loss could be attributed to isomerisation and oxidative degradation of structure and bioactivity of carotenoids, such as β-carotenoids and lycopene, which were due to the light exposure, presence of oxygen, high temperature storage, presence of peroxides, type of packaging and storage times. In addition, Dutta, Chaudhuri & Chakraborty (2005), reported that lipooxygenase activity and other enzymes, and coupled oxidation with lipids also promote degradation of carotenoids. However, the transfer of lycopene into micelles of NP was significantly different ($p<0.05$) with its dried extract form. The bioaccessibility of lycopene for both dried extract forms of the two microalgaes were found to be higher when compared to their powdered forms.

Bioaccessibility of vitamin E

Figure 3 shows the proportion of ingested α-tocopherol which becomes available to the body for absorption after simulated digestion process was carried out for the CP, CE, NP, and NE. The figure shows that CE has the highest bioaccessibility of α-tocopherol (72.1 $\pm$ 1.2 g kg^{-1}), followed by CP (56.3 $\pm$ 0.1 g kg^{-1}), NE (48.4 $\pm$ 1.0 g kg^{-1}), and NP (45.9 $\pm$ 1.0 g

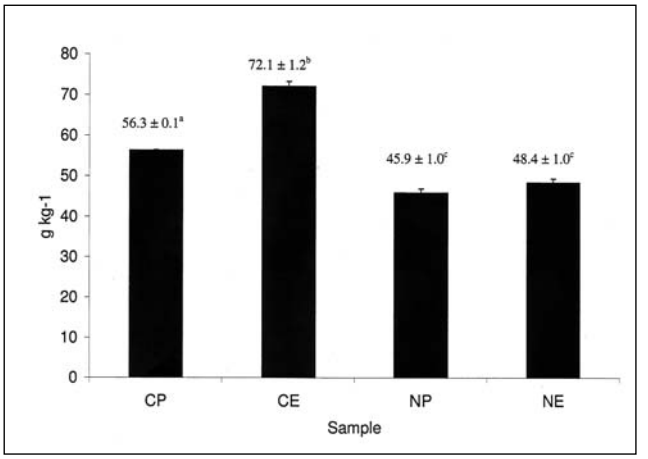

Figure 3. Bioaccessibility of alpha-tocopherol in powdered and dried extract form of *Chaetoceros calcitrans* and *Nannochloropsis oculata* microalgae
Note: Values with different superscripts indicate significant difference at 0.05 level

kg^{-1}) respectively. These results indicate that *C. calcitrans*' bioaccessibility of α-tocopherol is higher when compared to *N. oculata*, regardless of powdered form or dried extract form.

One way ANOVA test showed significant differences ($p<0.05$) between the CP, CE, NP and NE in terms of α-tocopherol's bioaccessibility. There were no significant difference between NP and NE. However, there were significant differences between both the powdered form and the extract form of *C. calcitrans* (CP and CE). The bioaccessibility of α-tocopherol for both the dried extract forms of the two microalgaes were found to be higher compared to their powdered forms (Figure 3).

Figure 4 shows that β-tocopherol was not detected in both types (powdered form and extract form) of *C. calcitrans*. However, between the two forms of *N. oculata* samples, the extract form had a higher bioaccessibility of β-tocopherol (55.7 ± 0.1 g kg^{-1}) compared to the powdered form (53.0 ± 0.6 g kg^{-1}). There was no significant difference found in β-tocopherol bioaccessibility between NP and NE. This showed that the content of β-tocopherol transferred into micelles was similar between the powdered form and the extract form of *N. oculata*. Lastly, both γ- and δ-tocopherol were not detected in both microalgae.

The predominant form of vitamin E in the body is α-tocopherol comprising over 90 % of vitamin E (Burton & Traber, 1990). This form has been widely researched owing to its antioxidant and non-antioxidant functions. From the results obtained, both the *N. oculata* and *C. calcitrans* microalgae show potential α-tocopherol absorption in the gastrointestinal tract with higher amounts found for the extract form compared to the powdered form. Similarly with carotenoids, the higher bioaccessibility of dried extract forms may be due to their disrupted form compared with powdered forms. Thus, the matrix of the dried extracts may have been disrupted, exposing larger surface areas to the reaction of enzymes.

According to Borel (2003), the food matrix has a marked effect on highly lipophilic food microconstituents (HLFM). At the same time, different locations of HLFM in foods, and different physiochemical states of HLFM in different foods, and different kinds and amounts of absorption effectors such as fibres (Riedl *et al*., 1999), fats (Borel *et al*., 1998) and phytosterols (Richelle *et al*.,

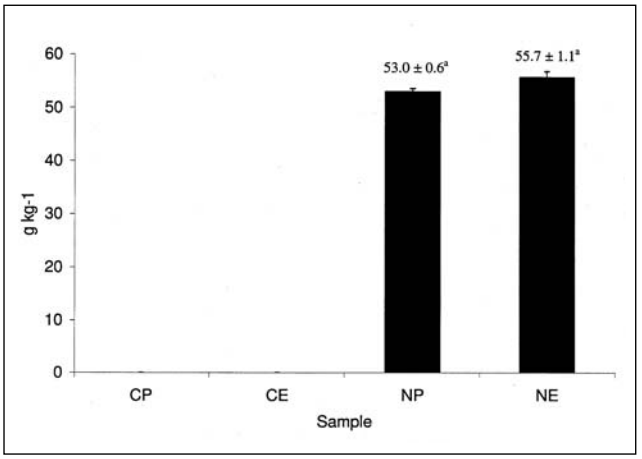

Figure 4. Bioaccessibility of beta-tocopherol in powdered and dried extract form of *Chaetoceros calcitrans* and *Nannochloropsis oculata* microalgae
Note: Values with different superscripts indicate significant difference at 0.05 level.

2004) may affect the bioavailability of vitamin E. The total lipids found in *N. coulata* and *C. calcitrans* was 18.4% and 23.8% respectively (Rebolloso-Fuentes *et al*., 2001; Sánchez-Saavedra & Voltolina, 2006). The higher bioaccessibility of α-tocopherol in both CP and CE compared to NP and NE might be due to higher lipids found in *C. calcitrans* compared to *N. coulata*, which increased the amount of potential α-tocopherol for absorption. Besides, it was also reported that the efficiency of α-tocopherol absorption decreases when the amount of α-tocopherol given to experimental animals increases (Traber *et al*., 1986). The amount of α-tocopherol was found to be higher in *N. oculata* compared to *C. calcitrans* and this might also be another reason why the bioaccessibility of *C. calcitrans* was higher compared to *N. oculata*.

CONCLUSION

Micoalgae in dried extract forms was found to have higher bioaccessibility than the powdered form. The disruption of the food matrix could be the main factor that contributed to the higher bioaccessibility of nutrients shown by the dried extract forms. The fact that bioaccessibility measured for tocopherols was higher than those measured for β-carotene and lycopene further supports previous studies that tocopherols are better absorbed than carotenoids. Besides, the differences observed between *N. coulata* and *C. calcitrans* could be due to the cell wall structure that differs between a green algae and a diatom. The present protocol, although it cannot be considered as entirely physiological (that is, mastication, gastric pH), provides some quantitative measurement for carotenoids and tocopherols.

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