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EVALUATION OF STRATEGIES FOR PRESERVATION OF MICROALGAE *Chlorella*

TRABAJO FIN DE MÁSTER UNIVERSITARIO EN GESTIÓN DE LA SEGURIDAD Y CALIDAD ALIMENTARIA

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EVALUATION OF STRATEGIES FOR PRESERVATION OF MICROALGAE *Chlorella*

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RESUMEN

La biomasa obtenida a partir de microalgas, como la *Chlorella*, es utilizada en la elaboración de productos dietéticos, suplementos alimenticios y productos farmacéuticos, por lo que su demanda actual es creciente. Sin embargo, en muchos casos los lugares de producción se encuentran muy alejados de los consumidores dispuestos a pagar por ella. Habitualmente, la forma de distribución del producto es en formatos en polvo, siendo el secado responsable de la pérdida de propiedades nutricionales y el aumento de costes de producción. Por ello, el objetivo de este estudio es evaluar diferentes estrategias de conservación de microalgas *Chlorella* (tratamiento térmico de esterilización, material de envasado, acidificación y exposición a la luz) analizando cambios en el contenido en humedad, actividad de agua, contenido en sal, cambios en el color y crecimiento microbiano durante dos meses de almacenamiento a diferentes temperaturas. Los resultados obtenidos ponen de manifiesto que el tratamiento térmico modificó considerablemente el color de las muestras sin diferencias significativas por efecto de la exposición a la luz o el material de envasado, mientras que la adición de ácido cítrico preservó el color especialmente a bajas temperaturas. La acidificación de las microalgas con un 3.5% de ácido cítrico y envasadas a vacío parece ser el tratamiento recomendable de conservación sin necesidad de utilizar bajas temperaturas de almacenamiento. El envasado en vidrio o en material plástico no influyó significativamente sobre los parámetros analizados.

PALABRAS CLAVE: microalgas, Chlorella, tratamiento térmico, acidificación, tipo de envasado, temperatura almacenamiento.

RESUM

La biomassa obtinguda a partir de microalgues, com la *Chlorella*, és utilitzada en l'elaboració de productes dietètics, suplements alimentaris i productes farmacèutics, per el que la seua demanda actual és creixent. No obstant, en molts casos els llocs de producció es troben molt allunyats dels consumidors disposats a pagar per ella. Habitualment, la forma de distribució del producte és en format pols, sent la deshidratació la responsable de la pèrdua de propietats nutricionals i de l'augment de costos de producció. Per això, l'objectiu d'este estudi és avaluar diferents estratègies de conservació de microalgues *Chlorella* (tractament tèrmic d'esterilització, material d'envasament, acidificació i exposició a la llum) analitzant canvis en el contingut en humitat, activitat d'aigua, contingut en sal, canvis en el color i creixement microbià durant dos mesos d'emmagatzemament a diferents

temperatures. Els resultats obtinguts posen de manifest que el tractament tèrmic va modificar considerablement el color de les mostres sense diferències significatives per efecte de l'exposició a la llum o el material d'envasament, mentres que l'addició d'àcid cítric va preservar el color especialment a baixes temperatures. L'acidificació de les microalgues amb un 3.5% d'àcid cítric i envasades a buit pareix el tractament recomanable de conservació sense necessitat d'utilitzar baixes temperatures d'emmagatzenament. L'envasament en vidre o en material plàstic no va influir significativament sobre els paràmetres analitzats.

PARAULES CLAU: microalgues, Chlorella, tractament tèrmic, acidificació, tipus d'envasament, temperatura emmagatzenament.

ABSTRACT

The biomass obtained from microalgae, such as *Chlorella*, is used to make dietary products, supplements and pharmaceuticals so their demand has recently increased. However, in most cases, there are produced very far from the places where consumers are willing to pay for them. One of the most usual form of distribution is as dry product. However, in this format algae loss some of their nutritional properties and it implies a high cost of production. Therefore, the aim of this study was to evaluate different strategies (thermal sterilized treatment, material of packaging, acidification and light exposure) to preserve microalgae *Chlorella* to distribute it properly on moisture content, pH, water activity, salt content, changes in color and microbial growth for two months of storage at different temperatures. The obtained results showed that color was modified considerably by the thermal treatment, without influence of light exposure and type of package, whereas citric acid preserved it especially at low temperature of storage. Besides, the acidification of microalgae with 3.5% of citric acid and vacuum packaging would be the recommended treated without need of cold storage. Packaging in jars or bags did not implied changes in any of the parameters analyzed.

KEYWORDS: microalgae, Chlorella, thermal treatment, acidification, packaging type, storage temperature.

1. INTRODUCTION

The massive production of microalgae was first carried out by Germany during the II World War in order to obtain lipids to use them as a source of biofuel. After the II World War, microalgae biomass started to be considered as a supplement able to replace conventional animal or vegetable proteins to direct consumption of cattle or humans, shorting the inefficient protein chain food. Thus, from 1948, a group of scientifics in Carnegie Institution of Washington performed the first systematic study which establishes the scientific fundamentals of massive culture of microalgae. The aim of this project was to use green microalgae *Chlorella* for large scale food production (Burlew, 1953).

Chlorella, which belongs to the phylum Chlorophyta, is a green microalgae with a diameter of 2–10 μm. This microalgae contains a single chloroplast, is unicellular, coccoidal, and nonmotile and is widely distributed in fresh, brackish and marine water (Nurachman et al., 2015).

Microalgae biomass was named by the Food and Agriculture Organization of the United Nations (FAO) as "superfood" because of its high nutritional power, not only for their protein levels (approx. 50% dry matter) easily digestible due to the complete amino acid profile, but also because of essential components for the metabolism, such as minerals, vitamins, antioxidants and polyunsaturated fatty acids (especially Omega 3 and 6) (Buggypower, 2015).

It is a source of ß-glucan that is an active immunostimulator, reducer of blood lipids, and a free radical scavenger (Avani et al., 2010). Also, it has a 1-4% chlorophyll, pigment that detoxifies the body of heavy metals and pesticides (Se-Kwon, 2015).

It is important to highlight that microalgae contain polyunsaturated fatty acids (PUFAs), two of the most abundant fatty acids are linoleic acid (LA) and alpha-linolenic acid (ALA). Specifically, arachidonic acid Omega 6 can be synthesized by humans from LA and essential acid Omega 3, such as DHA and EPA, from ALA (Solana et al., 2014). However, plant, animals and humans are not as good as microalgae synthesizing long chain Omega 3, such as EPA and DHA. Traditionally, they have been obtained from fish and fish oils, but safety issues have arisen because of the accumulation of toxins in fish (Avani et al., 2010). For that reason, we must consider that microalgae are the initial EPA and DHA producers in the marine food chain and they can reach much higher EPA and DHA contents than other possible sources as some fishes or soybean (Adarme-Vega et al., 2012).

DHA is a dominant fatty acid in neurological tissue, constituting 20-25% of the total fatty acid in the gray matter of the human brain and 50-60% in retina rod outer segments. It is also abundant in the heart muscle tissue and sperm cells (Se-Kwon, 2015). On the other hand, EPA is suggested to prevent human diseases such as arrhythmia, atherosclerosis, cardiovascular diseases, blood platelet aggregation, and several carcinomas (Avani et al, 2010).

The moderate ingest of EPA and DHA has a beneficial effect on mental disorders (Sánchez-Villegas et al., 2008) as depression, also a lower blood

level of EPA and DHA were related to callous-unemotional traits in adolescent boys with attention deficit hyperactivity disorder (Gow et al., 2013).

It is noteworthy that in Europe brain disorders costed €798 at 2010 (Olesen et al., 2012) and chronic diseases, including cardiovascular diseases, are estimated to cost to the EU economy €700 billion annually (European Heart Network, 2015). Therefore, the search of new ways to introduce these PUFAs in diet could mean an important form to reduce diseases expenses.

Additionally, microalgae include a beneficial effect on the environment, because it releases oxygen while it consumes 1.87 kg of CO_2 per kg of dry biomass produced (Buggypower, 2015; Solana et al., 2014), whereas fruit trees consume only 0.50 kg of CO_2 per kg of dry biomass (Espada, 2013).

Despite the fact that microalgae have so many advantages for different uses, it must bear in mind that their production imply several problems, due to high installing and operating costs, huge difficulty in controlling the culture conditions, contamination bacteria or alien algae and unstability of light supply and weather (Yen et al., 2013). Moreover, the production location is far from consumption sites and, for that reason, there is a challenge to preserve their beneficial features during transport with an adequate shelf life to guarantee their successful commercialization. Currently, they can be found in specialized markets but in a dehydrated format and consequently, their production costs are even higher.

In consideration of all the above, the aim of this study was to assess different strategies (thermal sterilized treatment, material of packaging, freezing or cooling, reduced pH and light exposure) to preserve microalgae produced by a factory located in Madeira in order to make possible their transport to the points of sale. For that purpose, moisture content, pH, water activity, salt content, changes in color and microbial growth have been analyzed for two months of storage.

2. MATERIALS AND METHODS

2.1. Raw material

Microalgae samples were delivered frozen in polyethylene bags by the Buggypower Company, which has a production plant in Madeira Island (Portugal), where microalgae grew in vertical photobioreactors. In turn, dehydrated green and red commercial algae were used to compare their characteristics with microalgae. For that, the commercial algae were rehydrated using an algae:water ratio of 5:500 (w/w) for 20 minutes using the manufacturer's recommendation. Then, algae were drained.

2.2. pH adjustment of microalgae

In part of the microalgae samples, citric acid was added at different concentrations (2.5% or 3.5%) to check the effect of this preservative in its shelf life.

2.3. Thermal treament

Part of the microalgae were sterilized in an autoclave at 115ºC for 15 minutes to extend their shelf life.

2.4. Packaging and storage of microalgae

Samples packaged in jars or in bags of bioriented poliamide/polipropilene thermal resistant (Bolsemack S.L.) were vacuum sealed when they were stored at room temperature, whereas samples stored at 4ºC or -18ºC were sealed without removing the air in the headspace of the package. Besides, in samples stored at room temperature the influence of light was also controlled by leaving part of them in close chambers and other samples exposed to light. Table 1 gives a description of all the combinations used in the storage of microalgae along with the notation for each case. Microalgae samples were stored for 2 months.

TABLE 1. Description and notation of the treatments for stabilization of microalgae

STORAGE CONDITIONS	DESCRIPTION	NOTATION
FRIDGE (4ºC)	BAG- 3.5% citric acid – Without thermal treatment	Bag 3.5 4C
	BAG – 2.5% citric acid – Without thermal treatment	Bag 2.5 4C
	BAG – Without citric acid – Without thermal treatment	Bag 4C
	JAR- Without citric acid – Without thermal treatment	Jar 4C
ROOM TEMPERATURE – WITHOUT LIGHT	BAG - 3.5% citric acid - Without thermal treatment	Bag 3.5 RT
	BAG - 2.5% citric acid - With thermal treatment	Bag 2.5 TT RT
	BAG – Without citric acid – With thermal treatment	Bag TT RT
	JAR - Without citric acid – With thermal treatment	Jar TT RT
ROOM TEMPERATURE – WITH LIGHT	BAG – 3.5% citric acid – Without thermal treatment	Bag 3.5 RT L
	BAG – 2.5% citric acid – With thermal treatment	Bag 2.5 TT RT L
	BAG – Without citric acid - With thermal treatment	Bag TT RT L
	JAR - Without citric acid - With thermal treatment	Jar TT RT L
FREEZER (-18ºC)	BAG- Without citric acid – Without thermal treatment	Bag -18C

2.5 Analytical determinations

The analytical determinations of moisture content, pH, a_w and color measurements were performed at day 0, 30, 45 and 60 after packaging. Additionally, microbiological analysis of mesophilic aerobics, molds and yeasts were performed to analyze the evolution of microalgae with a frequency of 30 days during two months. The determination of the effect of citric acid on the pH of microalgae was performed initially. By contrast, the analysis of sodium chloride was performed at the end of the study period. Below it is shown the description of the employed methods:

2.5.1 MOISTURE

Moisture determination was performed following an adaptation of method 934.06 (AOAC, 2000), the sample was heated under -0.8 bar of pressure and 60ºC in a vacuum oven (JP Selecta model Vaciotem-T), and the loss of weight was used to calculate the moisture content of the sample.

The effect of using a vacuum oven is to minimize cold spots as well as to exhaust moisture in the interior air (Nielsen, 2010). The results of moisture content were given in g of water per g of microalgae.

2.5.2 WATER ACTIVITY (A_W)

Measurements were performed with a Water activity meter (AQUALAB, model 4TE) at 25 °C.

2.5.3 POTENTIAL OF HYDROGEN (pH)

Measurements were performed in duplicate using a pH meter with a contact electrode (METTLER TOLEDO SevenEasy model), previously calibrated with buffer solutions of pH 4.00 and 7.00 at 25 ° C.

2.5.4 SODIUM CHLORIDE (X_S)

Measurements were carried out in triplicate with a Chloride Analyzer (CORNING, model 925) using a combined acid buffer solution as a support electrolyte that maintain the correct pH for the complete cycle of titrations and a colloid to prevent precipitation. This equipment was previously calibrated with a standard solution of 200 mg /l Cl at 25 ° C.

Aliquots of microalgae samples were diluted in a ratio 1:200 (w/w) and titrated in the equipment. The results of sodium chloride content were given in g of ClNa per g of microalgae.

2.5.5 COLOR MEASUREMENTS

Surface color was measured with a spectrocolorimeter (Minolta, modelo CM-3600d) by registering reflectance and using the small lens. The spectrocolorimeter was calibrated and measurements were done in triplicate over each surface of samples placed in cuvettes of 1-cm thickness. Color was recorded using the CIE-L*a*b* uniform color space (L*a*b*) considering the observer 10º and the illuminant D65.

2.5.6 MICROBIOLOGICAL ANALYSIS

Microbiological analysis of mesophilic aerobic, molds and yeasts were performed to the different treatments of treated microalgae at day 30 and day 60 of storage. The serial dilutions of samples were seeded using the pour plate technique by duplicate. Aerobic mesophilic were analyzed using plate count agar (Scharlau, 01-161-500, Barcelona, Spain) and incubated at 35±2.0°C for

48 hours. Molds and yeasts were analyzed using Oxytetracycline Sabouraud Agar Base (Scharlau, 01-275-500, Barcelona, Spain) with a sterile Oxytetracycline selective supplement (Scharlau, 06-115LYO1, Barcelona, Spain) and incubated at 25±2.0°C for 5 days.

For aerobic mesophilic, plates containing between 25 and 250 colonies were considered (FDA, 2001), while in the case of yeasts and molds, plates containing between 0 and 30 colonies were counted. The results were expressed as log CFU/g.

2.5.7 STATISTICAL ANALYSIS

The statistical software Statgraphics Centurion XVI was used to evaluate the statistical significance of the different treatments applied in preservation of microalgae, storage time and different conditions of storage. Interactions of the factors studied were analyzed with a significance level of 95 % ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1 Initial characterization of algae

Table 2 shows the results of moisture (x_w), sodium chloride content (x_s), pH and water activity (a_w) of microalgae *Chlorella* and the two dehydrated commercial algae that were rehydrated for analysis. The results give evidence of the similar values of water activity in all cases, while water content was higher in rehydrated algae due to this process itself. Besides, pH was higher in Green algae. In terms of salt content, red algae had the lowest value, although in all cases they came from seawater, where the concentration of salt is around 3.5% (Park et al., 2011). The differences in the salt content were also declared in the label of the commercial algae since the Green type had 1.6 g of salt/g of dry matter and the Red type, 0.7 g of salt /g of dry matter.

TABLE 2. Initial characterization of algae

Type of algae	pH	a_w	x_w(g water/g algae)	x_s(g salt/g algae)
Green algae (commercial)	7.60	0.9938	0.9192 ± 0.0009^{b}	0.02107 ± 0.00016^{b}
Red algae (commercial)	6.97	0.9973	0.9425 ± 0.0036^{c}	0.00311 ± 0.00016^{a}
Microalgae (*Chrorella*)	5.75	0.9814	0.8406 ± 0.0007^{a}	0.02033 ± 0.00019^{b}

Equal letters indicate homogeneous groups.

3.2. Effect of citric acid on the pH of microalgae

One of the classic strategies used in food preservation is the reduction of pH. Most foodborne pathogens cannot grow at a pH less than 4.4 (Montville and Matthews, 2009). Foods with lower pH (value below 4.5) are not altered easily by bacterias, being more sensitive to alteration by yeasts and molds (Casp and Abril, 2003). For that, in this study, preliminary experiments were performed using citric acid to achieve a pH below 3.5 to ensure greater stability of microalgae, and the results are shown in Figure 1. As can be seen, with

2.5% of citric acid, samples reached a pH lower than 3.5, thus this was the first concentration of citric acid chosen to extend the shelf life of microalgae with mild thermal treatment. It was also decided to work with a concentrations of 3.5 % citric acid to see the influence of a lower pH on the stability of microalgae.

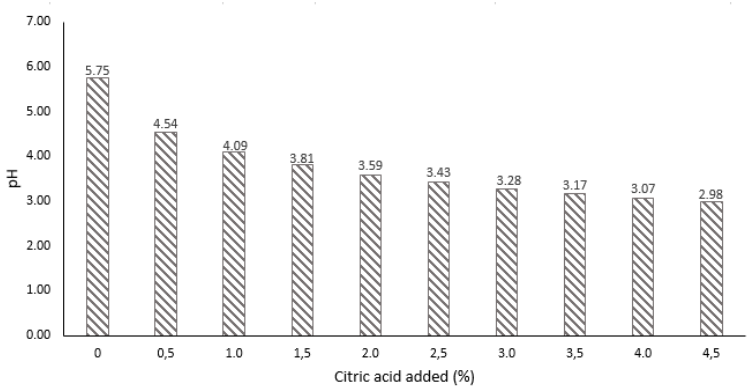


FIGURE 1. Effect of citric acid on the pH of microalgae

Furthermore, Figure 2 shows the values of pH of microalgae stored in different materials, temperature and depending on the thermal treatment and citric acid addition. In general, no factor affected the pH, in exception of the initial value of citric acid added, as it was expected.

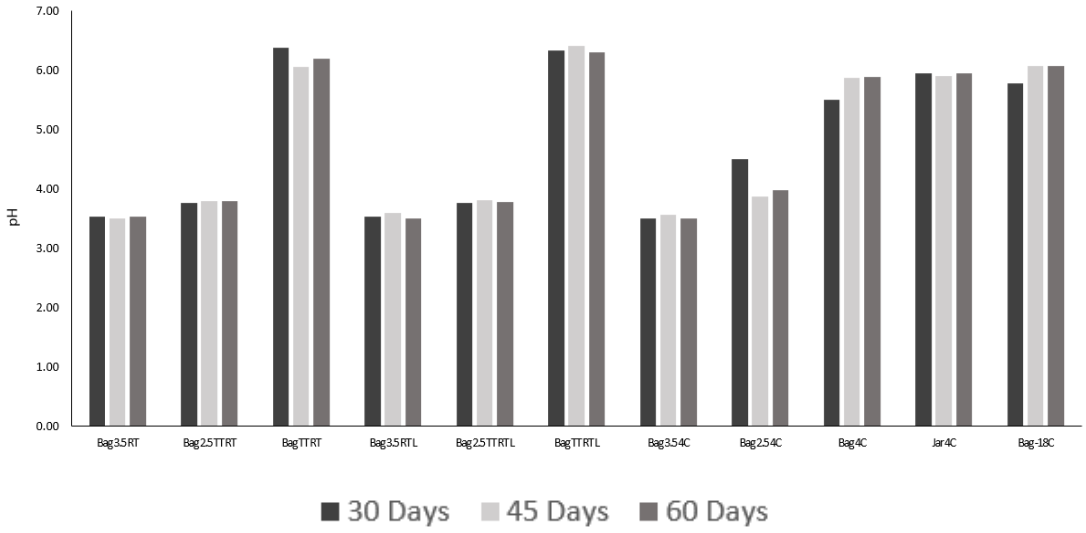


FIGURE 2. pH evolution throughout storage time

3.3 Moisture content and water activity

The values of water content in microalgae depending on the storage temperature, citric acid addition, exposure to light, packaging material and thermal treatment are given in Figure 3. In all cases, there was a reduction of the amount water with regard to the initial values of microalgae. Besides, the most remarkable fact is that the more citric acid concentration, the lower the

moisture content in microalgae which is in coherence with the increase of solids in the samples, although no differences between both concentrations of citric acid were observed at refrigerated storage. Overall, there was a slight decrease in moisture content throughout time, probably due to the water permeability of the packaging material. Thus, glass material, impermeable to water vapour, maintained better moisture content. In fact, the slight difference found among moisture content of microalgae packaged in jars or in bags stored at room temperature were not found when they were refrigerated since permeability depends on temperature. On the other hand, at room temperature, exposure to light had no significant influence on moisture content. At freezing temperatures, moisture loss in samples was slightly lower than when samples were stored in refrigerated conditions.

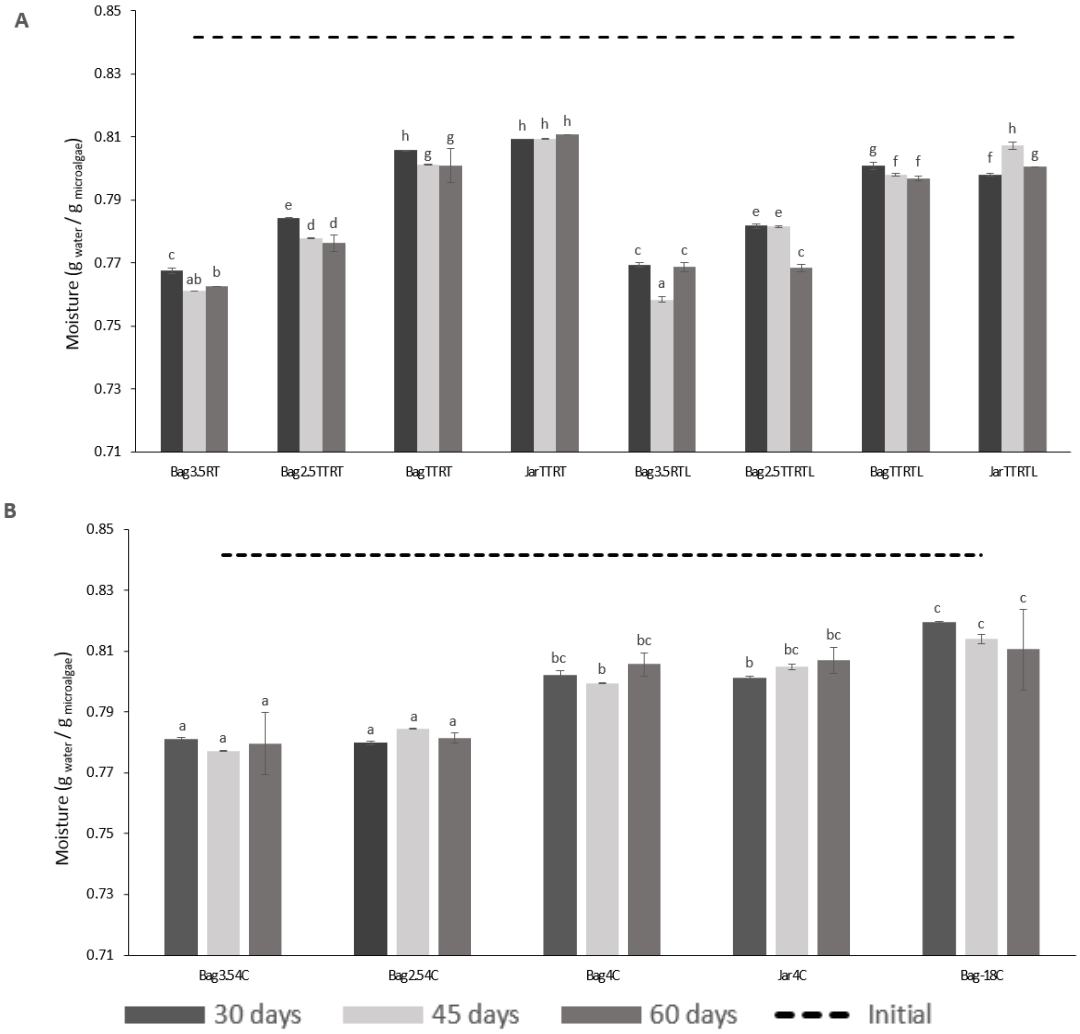


FIGURE 3. Moisture content of treated microalgae depending on the storage temperature and time. **A:** Storage at room temperature. **B:** Frozen or refrigerated storage.

Despite the differences in moisture content of microalgae according to the addition of citric acid, water activity remained quite similar to the raw material for all cases (0.9761±0.0037).

3.4 Sodium chloride content (x_S)

The amount of sodium chloride of treated microalgae after two months of storage is shown in Figure 3. In general, there was an increase of salt content with respect to the values of raw material, in coherence with the loss of moisture content before mentioned, without significant differences among the considered treatments.

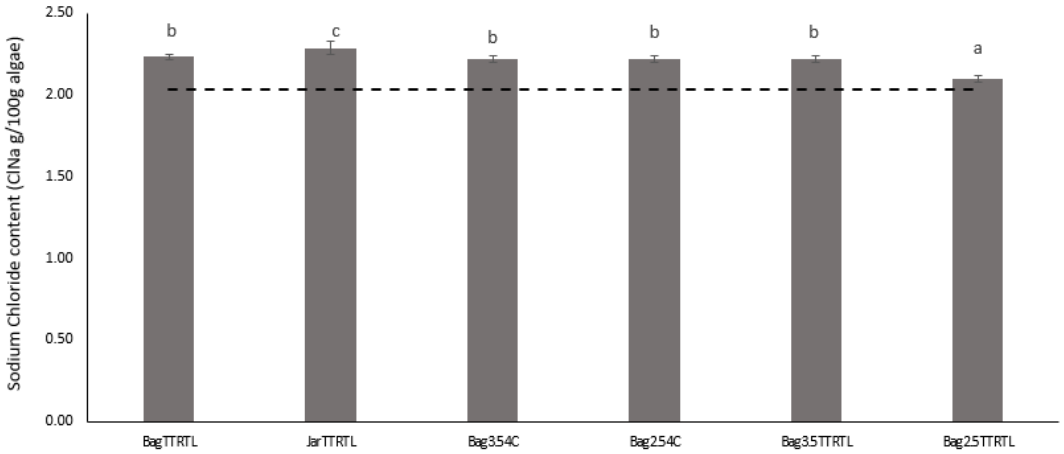
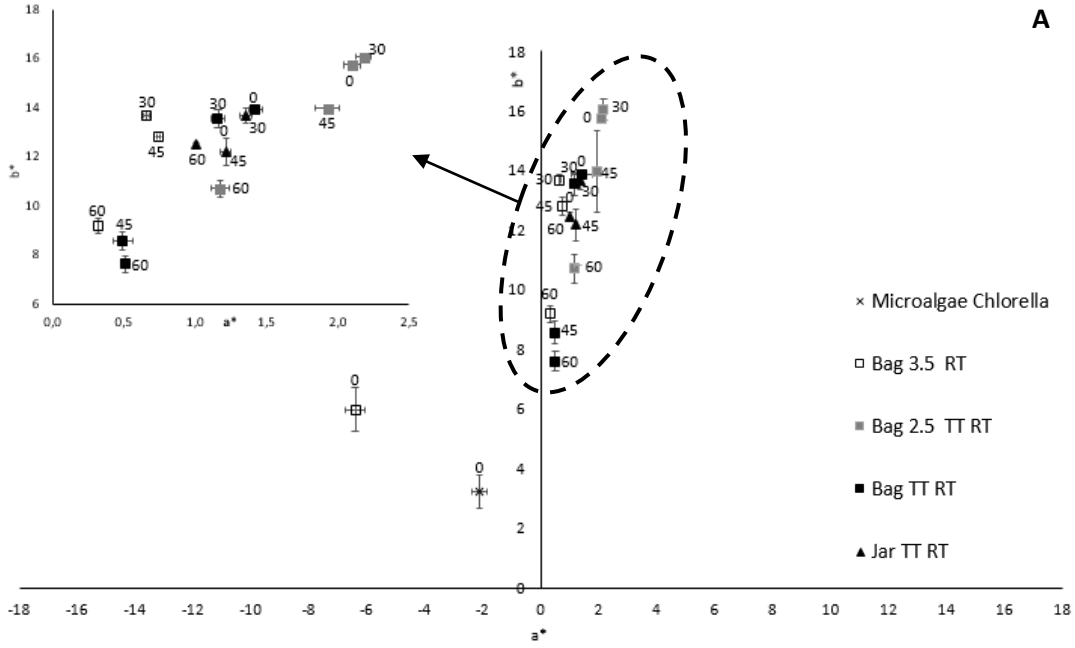


FIGURE 4. Sodium chloride content after two months of storage. Dashed line represents the value of sodium chloride content for raw material initially.

3.5 Optical properties

Figures 5 and 6 show the location of the coordinates b* and a* in the chromatic plane for microalgae stored at room or at refrigerated/frozen temperature, respectively.

It is noteworthy that the thermal treatment increased a* and b* coordinates, what implied a browning of the microalgae and a change in quadrant position of samples (from the second to the first one) in the chromatic diagram. Throughout storage time both coordinates decreased at room temperature. With regard to citric acid, initially it significantly reduced coordinate a* giving place to more intense green color. This greenish color was kept in microalgae stored at refrigerated/frozen temperature but not when they were at room temperature. In fact, the highest values of hue were for microalgae no treated with citric acid and stored at low temperature. Therefore, thermal treatment was the most responsible factor for the browning of microalgae as has been observed in previous studies because chlorophyll is almost no detectable after treatment with temperature higher than 40ºC in kiwifruit pulp (Schwartz et al., 1999). It is also important to bear in mind that heating caused the EPA and DHA content decreased significantly (Hadipranoto, 2005). The exposure to light did not mean a change in color of microalgae and consequently, they are suitable to be distributed in transparent package. Moreover, no differences in color of samples placed in bags or jars were found, so it seems easily and cheaper to use the plastic bag.



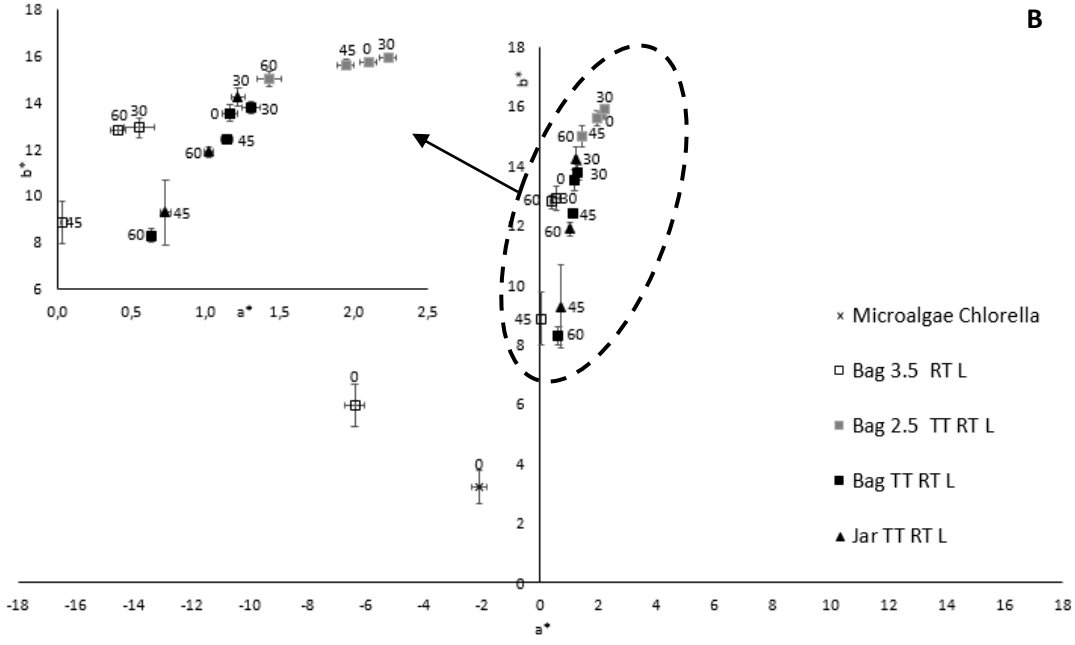


FIGURE 5. Chromatic planes b*-a* of treated microalgae stored at room temperature without exposure to light (A) or with light (B)

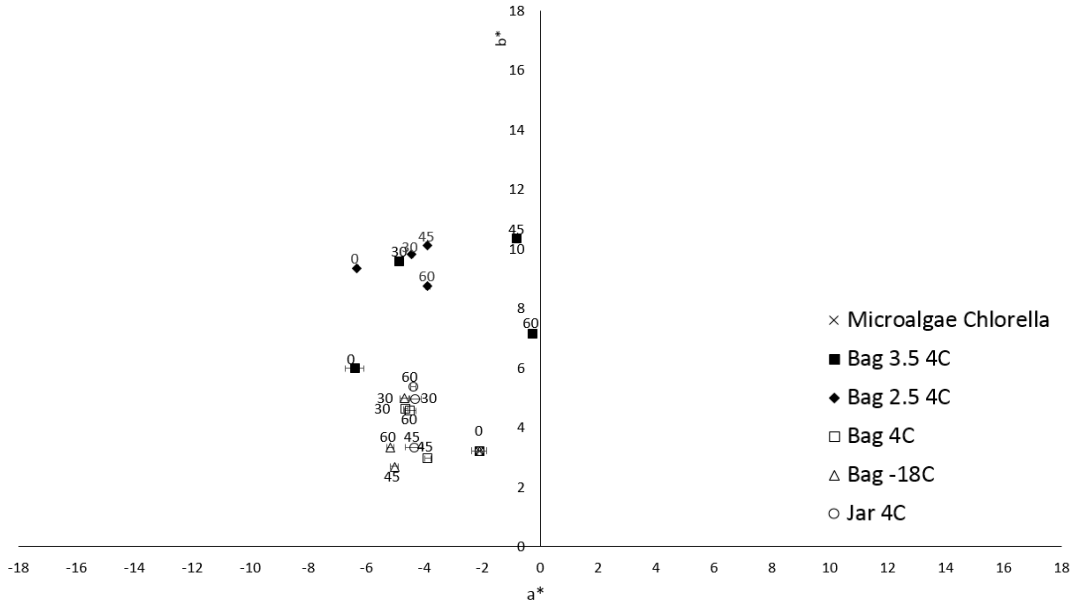


FIGURE 6. Chromatic planes b*-a* of treated microalgae stored at refrigerated/frozen temperature

The results of luminosity of treated microalgae are given in Figure 7. As a whole, this parameter changed less at room temperature than at refrigerated/frozen temperature. It is likely that the thermal treatment applied was responsible for this homogeneity. Also, at room temperature, the addition of citric acid contributed to a high luminosity at the end of storage. No significant differences were found by the influence of light exposure. In the case of microalgae stored at refrigerated/frozen temperature, luminosity was very similar to the raw material values till 30 days of storage. However, then there was a significant decrease in this parameter except for the microalgae treated with citric acid. The material of package did not affect the values of luminosity.

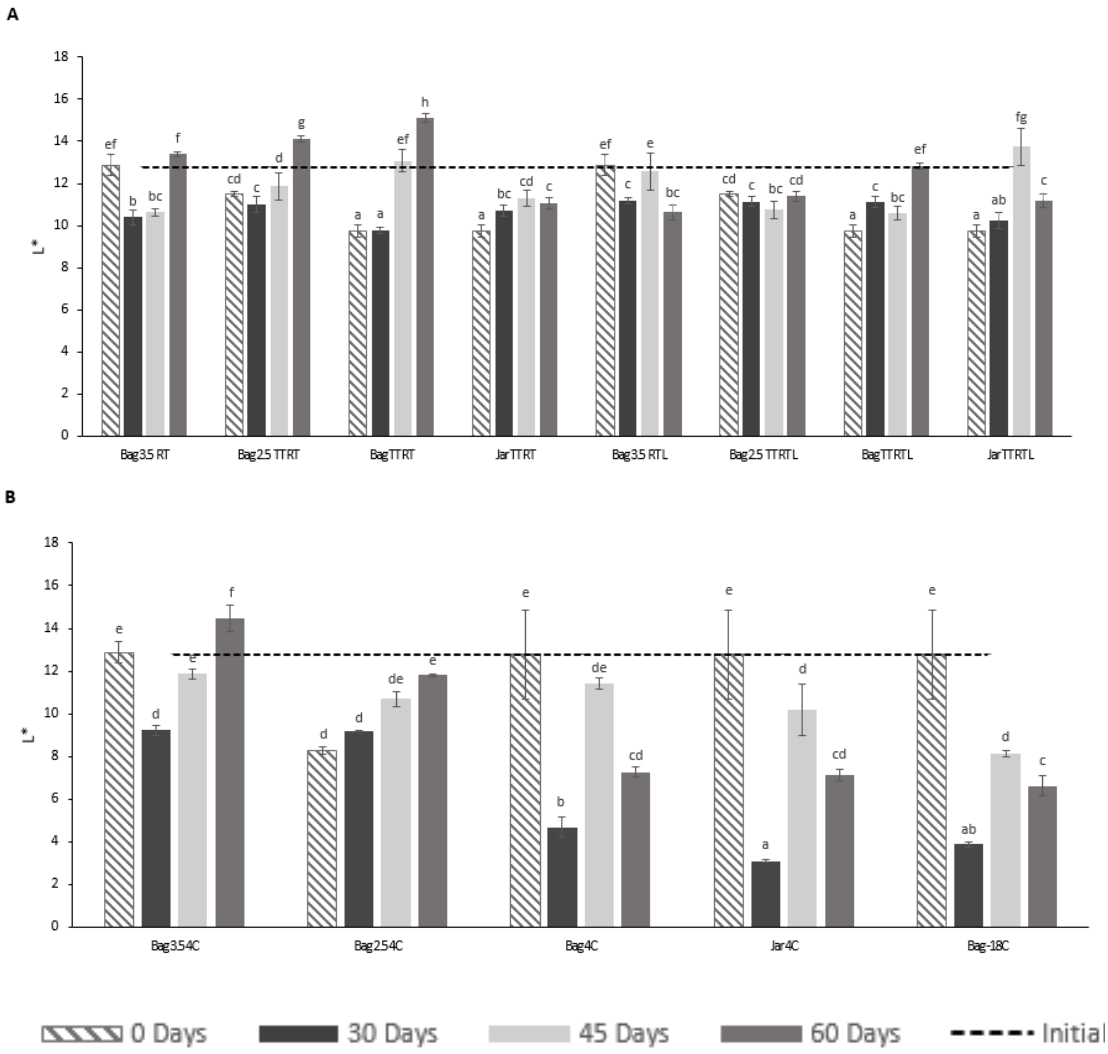


FIGURE 7. Luminosity of treated microalgae throughout time at room temperature (A) and at refrigerated/frozen temperature (B). Dashed line represents the initial value for raw material

3.8 Microbiology counts

The aerobic mesophilic and molds and yeasts counts of treated microalgae are represented in Figure 8. In the absence of legislation to these products, the acceptable limits for both types of microorganisms have been considered as indicated by the regulation (Spain, RD 3484/2000) concerning to hygiene standards for elaboration, distribution and merchandising of prepared foods. Concretely these limits are $1x10^6$ CFU/g for aerobic mesophilic on the expiry date. In case of molds and yeast, the maximum limit was $1x10^2$ CFU/g based on the criterion recommended for fruits and vegetables by Pascual and Calderón, 2000.

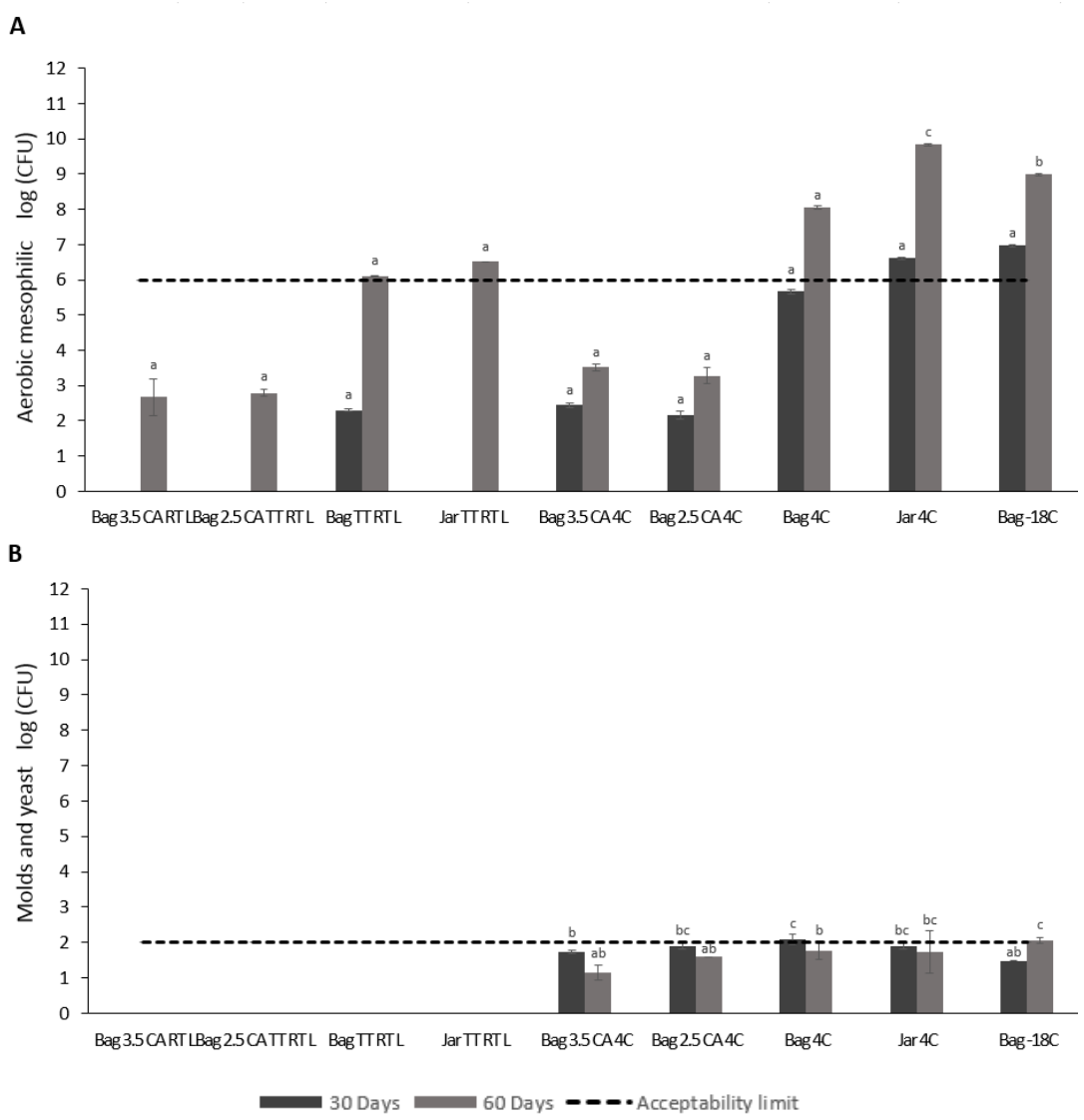


FIGURE 8. Microbiological content of treated microalgae depending on the storage temperature and time. **A:** Aerobic mesophilic counts. **B:** Molds and yeast counts.

According to these results, the addition of citric acid significantly reduced the growth of aerobic mesophilic in samples in the studied storage period. However, no significant differences in aerobic mesophilic growing were found between the two tested citric acid levels in microalgae stored at room temperature. In non-acidified samples the acceptable limit was overpassed between 30 and 60 days of storage, even in thermally treated samples. This could be related to the initial pollution level in the raw material or to the presence of spores. It is also important to point out that low temperatures in storage did not slow down the aerobic mesophilic growth. In addition, the absence of oxygen in containers stored at room temperature would be responsible for the slower growth of microorganisms.

The applied thermal treatment reduced the growth of molds and yeast in the analyzed storage time. Again low temperatures (4 and -18ºC) did not reduce the development of this type of microorganism, but without exceeding the

established threshold. Finally, in this case the citric acid did not improve the stability of microalgae. As was aforementioned, molds and yeast are less sensitive to the reduction of pH, being their minimum range to grow properly among 1.5-3.5 (Casp and Abril, 2003).

To sum up, although thermal treatment could mean a reduction of molds and yeast, it is not clear if this response if related to the absence of oxygen, since all thermally treated samples were vacuum packaged. Therefore, the addition of 3.5% of citric acid would be the recommended treatment to guarantee the maximum shelf life from the microbiology point of view.

4. CONCLUSIONS

Thermal treatment changed considerably the color of this product without influence of light exposure and type of package, while citric acid preserved it especially at low temperature of storage. In terms of microbial stability, the acidification of microalgae with 3.5% of citric acid and vacuum packaging would be the recommended treated without need of cold storage. In conclusion the best way to transport and distribute microalgae would be acidification and vacuum packaging in bioriented poliamide/polipropilene bags stored at 4ºC.

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