Natural Colorants Extracted from Microalgae with Potential Use in Food: Case Study of C-phycocyanins

C. García Gómez[#1], E. Garza Valverde[#2], J. Nápoles Armenta[#3], R. A. De Hoyos Cantú[#4]
[#]Autonomous University of Nuevo León, Campus of Agricultural Sciences, Faculty of Agronomy, Nuevo León, Mexico
[1]celestino.garciagm@uanl.edu.mx, [2]elizabeth.garzava@uanl.edu.mx, [3]juan.napolesrm@uanl.edu.mx, [4]ramon.dehoyos@alismty.com.

***Abstract* — The use of spirulina for the formulation of functional foods has increased in recent years due to its properties and health benefits. In addition, the use of spirulina phycocyanin is of interest for its functional properties in the human body, with health benefits ranging from anti-inflammatory action to its power as an anticancer agent. However, one of the problems with the use of phycocyanin in food is to optimize its extraction at maximum concentration and with good stability. In this work, a method of extraction and precipitation of phycocyanin with different salts was standardized, with the aim of optimizing the parameters of the extraction process (temperature, pH, agitation, amount of solvent and time) and precipitation (temperature of storage, precipitating salt concentration, and time) to maximize phycocyanin concentration, using the response surface method. The results of the proposed phycocyanin extraction and precipitation method managed to obtain concentrations of 0.378 mg/ mL and 0.47 mg/g of phycocyanins respectively, thus obtaining a natural pigment for incorporation into beverages.**

***Keywords* —phycocyanin, spirulina, microalgae, food, natural colorant.**

I. Introduction

Spirulina is a cyanobacterium, also known as Arthrospira, popularly recognized in the health food world as a high-protein supplement to the human diet. It is a photosynthetic organism that naturally grows in conditions of intense sunlight, high temperatures and a highly alkaline medium [1]. It is characterized by a high protein content, containing all the essential amino acids, although reduced amounts of methionine, cysteine and lysine, thus being inferior to animal proteins such as eggs and milk, but far superior to most vegetable proteins. In addition, it contains a high amount of polyunsaturated fatty acids (1.5-2%), vitamins B1, B2, B3, B6, B9, B12, C, D and E, and minerals such as potassium, calcium, chromium, iron, copper, magnesium, manganese, selenium and zinc [2]. Currently, the use of spirulina as an alternative source of protein enjoys great popularity, due in part to the increase in people in the world who follow diets free or reduced in consumption of animal protein. In Mexico, the percentage of people who follow vegetarian, flexitarian and vegan diets is 19%, 15% and 9%, respectively. Additionally, the functional foods market worldwide has a value of 161.49 million USD, with a projected annual growth of 7.9% [3].

One of the disadvantages of using Spirulina for food formulation is the formation of off-flavors in it, which may be due to cyanobacterial metabolites, such as geosmin and 2-methylisoborneol, which create "earthy" aromas, as well as to the presence of eicosapentaenoic and docosahexaenoic fatty acids, which impart a certain "fishy" flavor. Therefore, the use of phycocyanin extracted from spirulina is of interest to avoid the unpleasant notes described above.

Phycocyanin is part of the family of phycobiliproteins, which are pigments present in cyanobacteria that form light-absorbing complexes. Phycobiliproteins are made up of a heterodimer of α and β units, each of which is made up of one, two, or three chromophores called bilins. Within the phycobiliproteins are the types phycoerythrins, phycocyanins, allophycocyanins and phycoerthrocyanins. In Spirulina, the most important type is phycocyanin, specifically the C-phycocyanin type, which is composed of two α and β subunits with a hexameric conformation. The phycocyanin extracted from spirulina can be used in the formulation of foods due to its functional properties, among which are anti-inflammatory, antioxidant and anticancer, acting directly in the reduction of oxidative stress, apoptosis and inflammatory processes in in vivo models [4].

There are various extraction methods for phycocyanin from Spirulina or other cyanobacteria, but they mostly have disadvantages of low purity level and low storage stability [5]. Actually, it is interesting to evaluate a simple and economic method of extraction, evaluating the recovery of the pigment and its stability in storage for its use as a food ingredient. Therefore, the objective of the study was to validate an appropriate phosphate stirring extraction method to obtain stable phycocyanin and sufficient concentration, as well as an appropriate salt precipitation method to obtain phycocyanin in sufficient concentration for use as raw material in functional foods.

II. MATERIALS AND METHODS

A. *phycocyanin extraction*

For the extraction stage, dried spirulina (S. maxima) was used. The extraction procedure was based on the one described by Ying [6], using a phosphate buffer (pH 7.8), in this case KH_2PO_4, adjusted to a certain pH (6, 8 or 10), and where a certain quantity of sample (dried spirulina) and was subjected to stirring, time and temperature (Table 1). Once the extract was obtained, absorbance was measured at 615 and 652 nm to determine the amount of phycoerythrocyanin (PE), C-phycocyanin (CPC) and allo-phycocyanin (APC), as well as the total phycocyanins (TPC) present in it. The amount of each one of them (mg/ml) was determined by means of the following formulas:

$$CPC = (A_{615nm} - 0.474A_{652nm})/5.34 \quad (1)$$

$$APC = (A_{652nm} - 0.208A_{615nm})/5.08 \quad (2)$$

$$TPC = CPC + APC \quad (3)$$

TABLE I
EXPERIMENTAL CONDITIONS FOR PHYCOCYANIN EXTRACTION

Variable	**Experimental values**		
	min	***central point***	***max***
T (°C)	10	25	40
Agitation (RPM)	0	100	200
Biomass:solvent ratio	1:1	1:3	1:5
Time (min)	5	15	25
pH	6	8	10

Once the phycocyanin was extracted under the optimal conditions determined in the previous stage, its stability was evaluated at storage conditions of different temperatures (-20, -4, 5, 25, 40ºC) for a period of up to 4 days.

Additionally, the same procedure was carried out at a temperature of 72ºC for a period of 15 minutes, with measurements at times 0, 1, 2, 5, 10 and 15 minutes.

B. *Phycocyanin precipitation*

This stage consisted of carrying out the precipitation of phycocyanin previously extracted with phosphate buffer (with the optimal conditions obtained in stage A) from spirulina, testing various salts (ammonium sulfate, ammonium phosphate and sodium citrate) to find the most efficient salt in precipitation.

To do this, we started with phycocyanin extracted with a method described by Ying [6], using a phosphate buffer, in this case KH_2PO_4, adjusted to pH 7.8, and where a certain amount of sample (dry spirulina) was dispersed and subjected to at the following conditions, 40 °C, 150 rpm, sample/solvent ratio 1:5, 22 min and pH 7.8, which were validated in a previous experiment as the optimal point to maximize phycocyanin extraction from spirulina .

Subsequently, the following precipitating salts were added to the spirulina extract in different treatments and it was left to rest at refrigerated temperature for several hours.

- 65% ammonium sulfate
- 65% ammonium phosphate
- 10% sodium citrate

After that, the resulting product was centrifuged, the precipitate was separated, dried in an oven for 24 hours and dissolved in phosphate buffer to evaluate the sample at an absorbance of 615 nm and 652 nm and determine the amount of C-phycocyanin. (CPC) and allo-phycocyanin (APC), as well as the total phycocyanins (TPC) present therein as described in section A.

Subsequently, the salt that precipitated with the highest concentrations of phycocyanin was selected, as the one that presented fewer processing difficulties.

Finally, the optimization of the conditions (storage temperature, time and concentration of precipitating salt) was carried out to maximize the concentration of precipitated phycocyanin, with the selected salt (sodium citrate), see table 2. Once the phycocyanin was extracted with phosphate buffer, the resulting liquid was centrifuged, the precipitate was separated, dissolved in phosphate buffer and the absorbance was measured at 615 nm and 652 nm, with the procedure and equations described in the previous section to calculate the concentration of C-phycocyanin, allophycocyanin and total phycocyanins.

TABLE 2

CONDITIONS FOR PHYCOCYANIN PRECIPITATION

Variable	**Values experimental**		
	min	***Central point***	***max***
Storage time (h)	3	16	29
Storage temperature (°C)	5	15	25
Sodium citrate concentration (%)	5	10	15

All the data were analyzed by means of the response surface method through a Box- Behnken strategy, using the Design-Expert 13 software.

C. *Beverage application*

Once the optimal precipitation conditions were determined in the previous stage, we proceeded to use them to obtain a sufficient amount of phycocyanin and apply it in a dairy-based drink with the following formulations: 93.9% lactose-free whole milk, 0.1% xanthan gum, sugar 5%, phycocyanin 1, 2 and 5%. Subsequently, samples of each of the prepared beverages were taken and 15 people were tested (untrained panelists, consumers of milk-based products such as

drinkable yogurt or smoothies) to evaluate the sensory characteristics of the products in attributes such as appearance, texture, smell and taste by means of a 7-point hedonic test, in which 7 is the highest rating and 1 the lowest.

Subsequently, protein and fat determinations were made by the Kjeldahl ((NMX-f-608-NORMEX-2011) and Gerber butyrometric methods, respectively. (AOAC 2000.18), as well as L*a*b* color determination, using a Minolta CR410 colorimeter (Konica Minolta Co. Japan).

III. Results and Discussion

A. *Phycocyanin extraction*

The optimal extraction point of C-phycocyanin was at 40ºC, 151 rpm, 24.8 min, pH 7.7 and a sample-solvent ratio of 1:5, with a predicted concentration of 0.249 mg/mL. The model obtained managed to explain to a large extent the concentration of phycocyanin based on the mentioned variables (R_2 =0.8671), where the data obtained in the experimentation fit the predicted data well and remain close to it. According to ANOVA results, the temperature variables and the sample:solvent ratio were statistically significant with $p<0.05$. In the relationship between the temperature and the amount of solvent, the concentration of phycocyanin extracted increases as the temperature and the amount of solvent increase, leaving the maximum point of the interaction at the maximum temperature and the maximum amount of solvent, as can be seen in Fig. 1.

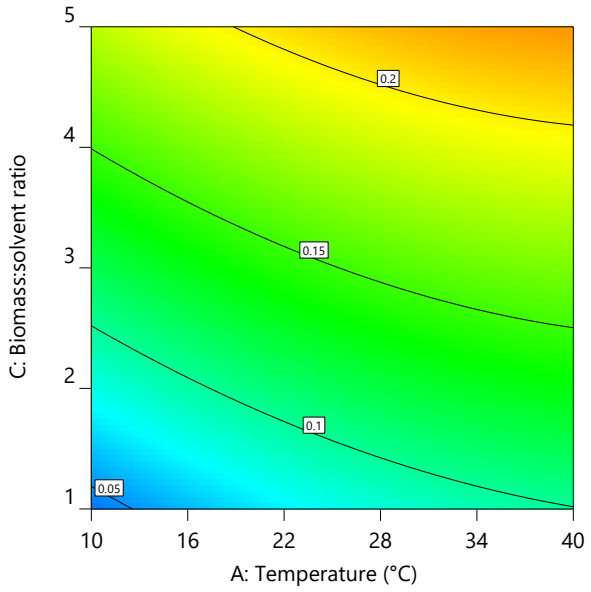

Fig. 1. Contour plot with the interaction of temperature and amount of solvent.

B. *Phycocyanin precipitation*

Sodium citrate was selected as the precipitating salt with the best performance in the precipitating salt evaluation system. The point at which the concentration of total phycocyanins was maximized coincided with the previous one, being 29 h, 25°C and 5% sodium citrate, a predicted maximum concentration of 0.470 mg/g of C-phycocyanin, 0.275 mg/g of allophycocyanin and 0.745 mg/g of total phycocyanins. In this case, the effect of temperature and storage time on the concentration of precipitated phycocyanin was notorious. The higher the storage temperature and time, the higher the concentration obtained at the end of phycocyanin. The effect of temperature on the speed of the reaction is notorious, as it has a directly proportional relationship with the precipitation of phycocyanin. In addition, a longer time also allowed a greater reaction of the precipitating salt with the dissolved phycocyanin, thus increasing its concentration in the final product. However, in the case of the sodium citrate concentration, an effect totally opposite to that of the other parameters was noted. The highest concentration of precipitated phycocyanin was obtained at the lowest citrate concentration, the former decreasing as the latter increases. This may be due to a supersaturation effect on the solution and a diluting effect on the precipitate. Since the highest concentration of phycocyanin obtained is achieved at 5% sodium citrate, increasing this does not have an effect on a greater precipitation of phycocyanin, but it does reduce its amount in the resulting product, as it has a higher concentration of phycocyanin. higher proportion of sodium citrate in the material. According to the ANOVA results, the citrate concentration has statistical significance in the phycocyanin precipitation. The model was able to explain to a large extent the phycocyanin concentration based on the mentioned variables (R^2 =0.8069). The fit of the model can be observed in the residual graphs and the comparison of these with the data predicted by the system (Fig. 2).

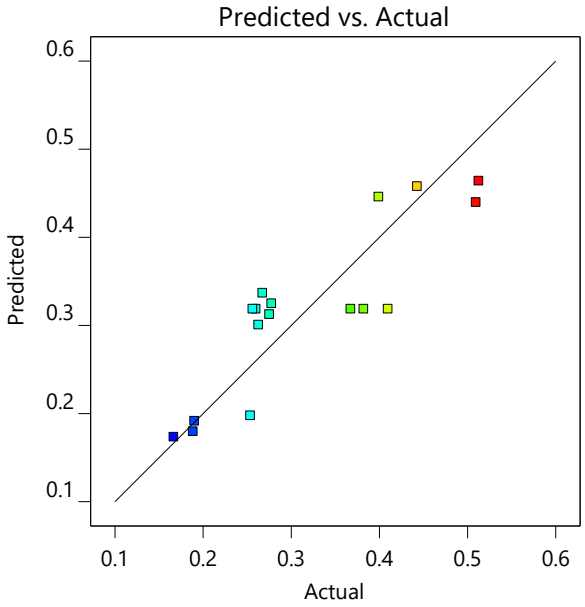

Fig. 2. Residual graph.

C. beverage application

The sensory analysis showed that the scores of all the attributes decreased as the level of use of phycocyanin in the beverage increased, impacting especially on appearance and aroma attributes. Therefore, the level of use of powdered phycocyanin should be between 1 and 2% in order to have a good level of acceptance by consumers and avoid defects in the appearance and taste of the beverage.

IV. Conclusion

It was possible to determine the conditions under which the concentration of extracted phycocyanin is maximized. The precipitation process managed to increase the concentration of phycocyanin from spirulina extract by 89% for C-phycocyanin and 97% for total phycocyanins. Taking into account that the concentration of phycocyanin increased, and therefore, the impurities decreased, this step manages to better adapt the material to be used as a functional ingredient in foods that seek an improved nutritional profile with functional properties in the organism. The application in a beverage allowed us to place the levels of use of the extract obtained in a specific range where there is a good acceptance by the consumer, thus avoiding higher levels where defects in the product may occur, focused mainly on the flavor and generating rejection by the market.

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