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Extraction and Use of Anthocyanins from Radish (*Raphanus Sativus* L Var Crimson Gigant) as a Natural Colorant in Yogurt

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ABSTRACT

Radish (*Raphanus sativus* L. var Crimson Gigant) contains significant quantities of glycosylated anthocyanins, it can be used as natural food colorants due to their high stability. These pigments have therapeutic and pharmacological characteristics that are associated to antioxidant activity. Furthermore, the pigments of radish are important for nutritional and medicinal uses, for all the benefits that they provide to human health. The objective of this research was to extract anthocyanins from the shell of radish in pure form, evaluate its antioxidant activity *in vitro* and then use it as a food coloring in natural yogurt. For sample extraction, the radish shell was separated by finely cutting it from the body of the radish, it was macerated with an acidified solution of methanol-water and evaporated by 80%. The extract was absorbed with an ion exchange resin, subsequently the anthocyanins were released in an acidified solution, finally the extracts were purified by reverse phase column chromatography. 14 fractions were obtained and were monitored by thin layer chromatography, afterwards antioxidant activity was evaluated by methods such as DPPH, ABTS and TBARS. A stable cherry powder was obtained, which was used as a yogurt colorant and was very well accepted by the judges.

Keywords Anthocyanins, Antioxidant Activity, *Raphanus Sativus* L. Var Crimson Gigant.

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I. Introduction

Anthocyanins have a flavonoid-type chemical structure, with two aromatic rings (A and B ring) and a heterocyclic ring (C ring) fused to the A ring. An example of anthocyanin is pelargonidone-3-O-glucoside (Fig. 1), which provides different coloration to plants (fruits and tubers).

Anthocyanins are water-soluble pigments that provide red, blue, and violet hues to eggplants, blueberries, purple corn, and geraniums, among others. [1], [2]. These chemical compounds that biosynthesize plants perform non-essential functions in them. At present, anthocyanins represent an option to replace artificial colorants, especially because they have a high degree of antioxidant capacity, as well as anti-inflammatory effects, benefits in heart problems, insulin resistance, and help in cancer problems. [3], [4]. The isolation of anthocyanins allows them to be used to provide color to chewing gums, beverages, candies, and jellies, demonstrating that it is possible to substitute synthetic colorants with natural

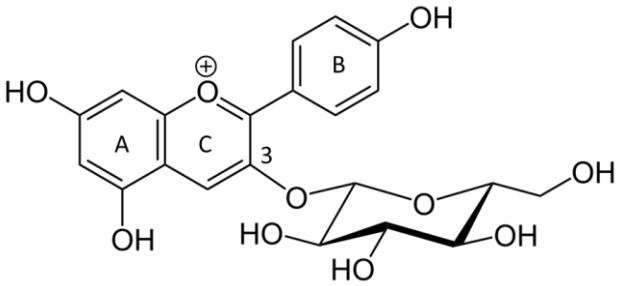

Fig. 1. Anthocyanin, derived from flavonoids, ring A, B, and C, with glycoside substituent at position 3.

ones, thus emerging the need to understand the chemical structure of these anthocyanin-type pigments. [5], [6]. The trend in today's society is to choose natural products with little processing, since in developing countries, synthetic dyes are still used, and their use in developed countries is already banned [7]. Although there are also safe synthetic colorants approved by the Food and Drug Administration (FDA). For this reason, new techniques have been used that enable the isolation of anthocyanins through natural pigments, achieving a high yield efficiency in the final product. Anthocyanins are recommended for their properties such as their biological activity, which plays a basic role in the prevention of pathologies such as the development of malignant tumors, diabetes, and neuronal and cardiovascular disorders [8]. In this research, anthocyanins were isolated from the epidermis of radish (Raphanus sativus L. var Crimson gigant) and their antioxidant activity was evaluated in vitro. Radish is an economical product in the market, it is grown all year round, and it provides a red coloration from its anthocyanins that are very stable and easy to isolate with ion exchange resins and its subsequent purification by reverse phase chromatography it also has a shelf life of approximately one year. The industry is not taking advantage of the biological potential of this tuber. Finally, to give a biotechnological application to the anthocyanins from radish, natural yogurt was pigmented, and its organoleptic characteristics were evaluated for its use as a natural colorant.

II. Materials And Methods

The radish (R. sativus) was grown in Santo Domingo Atoyatempan, Atlixco, Puebla, Mexico. To obtain the epidermis, they were washed with drinking water and the epidermis was removed manually.

A. Total Anthocyanin Content of Radish Epidermis

One gram of the epidermis was placed in a 25 mL Erlenmeyer flask, 20 mL of a methanol-water solution (85:15) acidified with 0.01% hydrochloric acid was added and ultrasound waves were applied for 20 minutes at room temperature.

After this time, the sample was filtered under vacuum and the aqueous phase was recovered in a flask. Subsequently, 200 µL of the filtered extract was taken and mixed with 1800 µL of a chloride buffer solution at pH 1.0 (hydrochloric acid/potassium chloride, 0.025 M). In addition, another 200 µL of the filtered extract was taken and mixed with 1800 µL of a buffer solution, but in this case an acetate buffer solution at pH 4.5 (acetic acid/sodium acetate, 0.4 M). With the Thermo Scientific GENESYS 10S UV visible spectrophotometer, absorbance was quantified at 510 and 700 nm, using distilled water as blank. The whole process was carried out in triplicate.

Total anthocyanin content was expressed as mg/g cyanidin 3-glucoside, following the differential pH method described by [9].

For the calculation, (1) and (2) were used:

$$\Delta A = (A_{max\,Vis} - A_{700nm})_{pH\ 1,0} - (A_{mas\,Vis} - A_{700nm})_{pH\ 4,5} \quad (1)$$

$$AT\ (mg/L) = \frac{\Delta T * PM * FD * 100}{£ * 1} \quad (2)$$

where
AT: Total anthocyanins;
ΔA: Change in absorbance;
PM: Cyanidin 3-glucoside: 449,2 g/mol;
FD: Dilution factor: (10);
£: Molar absorptivity of cyanidin 3-glucoside: 26900 L/cm mg.

B. Obtaining Crude Extract, Purification of Anthocyanins by Chromatography

6.67 kg (99 pieces) of R. sativus were used, obtaining 695 g of the fresh epidermis. Subsequently, the biological material was macerated for 24 hours in 500 mL of methanol, water (85:15), and 0.01% hydrochloric acid solution, placed in an Erlenmeyer flask, and ultrasound waves were applied for 20 minutes at room temperature. The resulting solution was filtered and evaporated to a final volume of 100 mL in a rotary evaporator with a heating bath at 35 °C, until an anthocyanin-rich extract was obtained.

Once the crude extract was obtained, consecutive washes were performed in a separatory funnel using a mixture of 30 mL of n-hexane and 30 mL of ethyl acetate as the organic phase until the organic phase showed no coloration.

The organic phase was discarded, and the aqueous phase was evaporated to 50 mL in a rotary evaporator with a heating bath at 35 °C.

The resulting solution was transferred to an Erlenmeyer flask with 20 grams of amberlite resin XAD7HP (20-60 mesh, Sigma-Aldrich) previously washed with distilled water; adsorption was performed at room temperature under constant agitation at (280 rpm) for 2 hours, avoiding exposure to light to ensure maximum anthocyanin adsorption. After the time elapsed, it was filtered under a vacuum, discarding the mother liquor which contains polar substances, but which do not have a positive charge. Finally, it was washed with 50 mL of deionized water, recovering the resin for desorption with 1.5 L of 2% methanol-hydrochloric acid. The colored solution was recovered in a ball flask and brought to dryness in a rotary evaporator with a heating bath at 35 °C and 32.8702 g of a sour cherry-colored powder was obtained.

Once the solvent-free extract was obtained, it was purified by column chromatography (30 cm × 3 cm), previously packed with 60 g of Sephadex (L-H 20. Sigma-Aldrich). 2 g of the extract (sour cherry powder) was dissolved with 5 mL of deionized water and poured into the column for elution using water, methanol, and hydrochloric acid in gradient. Fourteen fractions of 50 mL each were collected, and a thin layer chromatographic analysis (TLC) was performed from fractions 1 to 14, to observe the purity of the obtained fractions on silica gel 60, Alugram® Sil 0.20 mm UV254 plate.

A BAW (butanol, hydrochloric acid, and water) elution system (6:1:3 ratio) was used for CCF analysis. A UV lamp with a wavelength of 254 nm was used.

C. Neutralization of the Free Radical DPPH (2,2-diphenyl picrylhydrazyl)

A solution of the free radical DPPH, at a concentration of 133.3 µM dissolved in ethanol, was used. The degree of neutralization of the DPPH radical was done by measuring its

absorbance by spectrophotometry, at a wavelength of 517 nm and 50 µL of each isolated radish fraction was used, plus 150 µL of the DPPH solution, incubating at 37 °C for 30 minutes with orbital shaking. The support of this methodology is described by [10].

Equation (3) was used to determine the percentage inhibition of the DPPH radical of the isolated fractions:

$$\% = [(C - E)/C] * 100 \quad (3)$$

where
C=Average absorbance of the radical control;
E=Absorbance of the evaluated sample.
Samples were evaluated in triplicate

D. Free Radical Trapping Capacity by ABTS$^{\cdot+}$ Method of The Diammonium Salt Of 2,2'-Azino-Bis (3-Ethylbenzothiazolin-6-Sulfonic Acid)

Continuing with the process referred to by [11], which consists of mixing the 7 mM ABTS radical with 2.45 mM potassium persulfate (final concentration). Subsequently, 10 µL of each fraction (14 fractions) was taken and 990 µL of the diluted ABTS$^{\cdot+}$ solution was added. The antioxidant effect was monitored every minute up to 6 minutes and compared with the antioxidant capacity of Trolox and the results in this assay are expressed as Trolox equivalents.

(4) and (5) were used to calculate the percentage reduction of the ABTS$^{\cdot+}$ radical:

$$\% = (initial\ Abs - per\ minut\ Abs/initial\ Abs \times 100 \quad (4)$$

$$TEAC = \%\ reduction - 3{,}09777/4{,}76498 \quad (5)$$

Samples were evaluated in triplicate.

E. Iron-Induced Lipid Peroxidation in Rat Brain Homogenate (TBARS)

This technique is based on the determination of aldehydes formed by the degradation of lipids present in the sample. The most representative aldehyde obtained from such degradation is malondialdehyde (MDA), which forms a pink chromophore in the presence of 2-thiobarbituric acid (TBA).

To obtain the brain from male Wistar strain rats, euthanasia was applied in a CO_2 chamber according to the official Mexican standard; TECHNICAL SPECIFICATIONS FOR THE PRODUCTION, CARE AND USE OF LABORATORY ANIMALS (NOM-062-ZOO-1999). The whole brain was quickly dissected and washed with distilled water to remove blood. From the rat brain tissue, the homogenate was obtained, using 10 mL of phosphate-buffered saline (PBS) per gram of brain, at pH 7.4 and temperature of 5 ºC. The homogenate was centrifuged at 3000 rpm for 10 minutes. The supernatant was taken, and the protein content was determined by the method described by [11] and adjusted to 2.66 mg protein/mL with phosphate buffer, using bovine albumin as standard.

375 µL of supernatant was placed in 1.5 mL microtubes, added to 50 µL of EDTA, 25 µL of PBS solution, and 25 µL of the 14 fractions prepared for analysis were added, incubated at 37 ºC for half an hour. Peroxidation starts with the addition of 50 µL of freshly prepared FeSO4 (final concentration 10 µM), followed by incubation for another hour. The tubes were then immersed in an ice bath, 0.5 mL of TBA reagent was added, the mixture was heated at 94 °C for 30 minutes, then centrifuged at 12000 rpm for 10 minutes. The assessment of the extent of lipoperoxidation was defined in terms of MDA (malondialdehyde) production from the supernatant, by the development of the TBARS test described by [13].

After the time had elapsed, the tubes were cooled to room temperature and opened to release the pressure.

200 µL were taken in triplicate from each Eppendorf tube and transferred to a 96-well plate. The optical density was determined at λ=540 nm on an Elisa reader and intercalated to ranges on the standard curve to obtain the concentration of TBARS in solution, (µM concentration). Equation (6) was used in the calculation:

$$TBARS(\mu M) = A\ 540nm - 0{,}07386/0{,}09042 \quad (6)$$

To calculate the percentage inhibition (µM concentration), (7) was used:

$$\% = [(C - E)/C] * 100 \quad (7)$$

where
C: (Control): average optical density of + vehicle;
E: result obtained from the optical density of the samples.
The samples were evaluated in triplicate.

F. Stability Tests of Anthocyanins at Different Temperatures, pH and Natural Light

For temperature stability tests at 60, 70, 80 and 90 °C, 50 mg of the extract was taken, dissolved in 25 mL of 0.2 M chloride buffer at pH 3, 4, 5, 6 and 0.2 M phosphate buffer at pH 7.0. The solutions were placed in flasks and heated in a water bath for 1 hour. Aliquots were taken every 15 minutes and total anthocyanin quantification was performed. The heating time was determined according to the anthocyanin stability studies by [14].

To measure the stability of anthocyanins exposed to natural light, 50 mg of the extract was placed in transparent vials with lids and dissolved in 25 mL of 0.2 M chloride buffer at pH 3.0. They were exposed to sunlight for 9 days taking aliquots each day and measuring total anthocyanin content by differential pH. Samples were evaluated in triplicate.

G. Sensory Evaluation of Anthocyanin in Commercial Plain Yoghurt

1 g of anthocyanin extract was added to 120 g of natural yogurt. The pigment was incorporated by manual stirring and subsequently the properties of smell, color, flavor, and texture were compared and evaluated by sensory analysis in the commercial brands Alpura®, Lala®, Yoplait®, and Danone®. These samples were evaluated by 35 untrained judges (of legal age) who are regular consumers of the product. Participants signed a letter of informed consent.

H. Data Analysis

Data analysis was performed with the IBM SPSS Statistics 21 statistical package using ANOVA and Tukey's Honestly significant difference (HSD) test.

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III. Results

A. Total Anthocyanin Content of Radish Epidermis

The average anthocyanin concentration was 17.36±0.13 mg/g (mean ± S.E.) of cyanidin 3-glucoside.

B. Obtaining Crude Extract, Purification of Anthocyanins by Chromatography

From the 6.67 kg of R. sativus, 695 g of fresh epidermis were obtained. The 695 g of epidermis were macerated, and a final extract of 32.02 g was obtained with a yield of 4.61%, from which 2 g were taken, dissolved with 10 mL of deionized water, and purified by column chromatography, where 14 fractions were obtained (Fig. 2).

C. Neutralization of the Free Radical by DPPH (2,2-diphenyl picrylhydrazyl)

The antioxidant capacity contained in the 14 fractions of radish to reduce the DPPH radical was evaluated, and the percentage reduction (Fig. 3) was quantified by measuring the reduction in absorbance at 517 nm.

D. Free Radical Trapping Capacity by the ABTS$^{\cdot+}$ Method of The Diammonium Salt of 2,2'-Azino-Bis(3-Ethylbenzothiazolin-6-Sulfonic Acid)

The results show the average inhibition percentages of the 14 fractions at 6 minutes of reaction (Fig. 4). The fractions that showed the greatest effect on the ABTS radical were fractions 4 to 11, representing an inhibition percentage greater than 70%.

Table I shows the antioxidant activity of the ABTS test expressed as Trolox equivalents (TEAC), (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as it is used as a reference standard for antioxidant capacity.

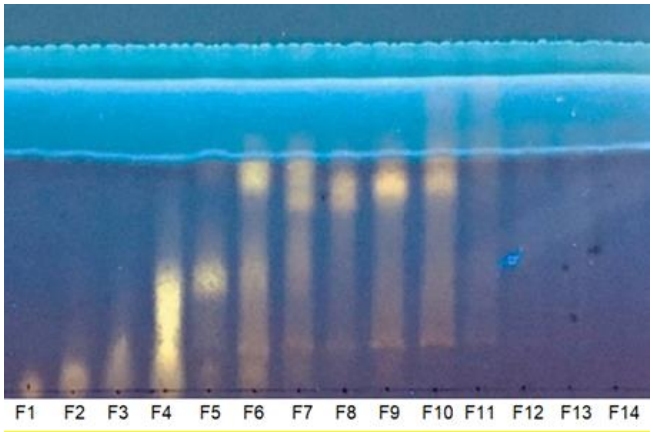

Fig. 2. Chromatoplate of fractions F1 to F14 under UV light at 356 nm.

TABLE I: Trolox Equivalent Antioxidant Activity (TEAC). Mean and Standard Error of Three Replicates

Fraction	µM Trolox/g	Fraction	µM Trolox/g
1	6.86 ± 1.27	8	17.1 ± 0.16
2	9.72 ± 0.41	9	17.1 ± 0.11
3	10.84 ± 0.28	10	18.26 ± 0.47
4	14.6 ± 0.24	11	15.43 ± 0.29
5	15.0 ± 0.21	12	12.2 ± 0.32
6	15.9 ± 0.25	13	7.91 ± 0.25
7	16.7 ± 0.22	14	5.26 ± 0.17

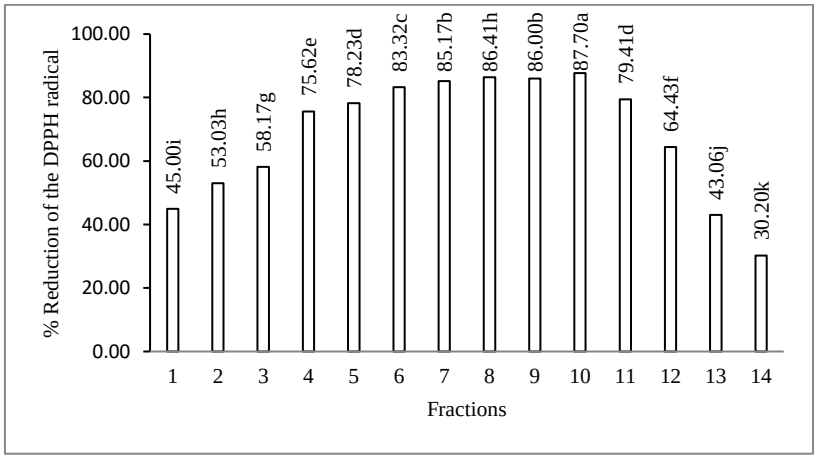

Fig. 3. Percent inhibition of the DPPH radical of fractions 1 to 14. Different letters indicate significant differences between antioxidant capacity averages (P≤0.05). The plot of the mean and standard error of three replicates.

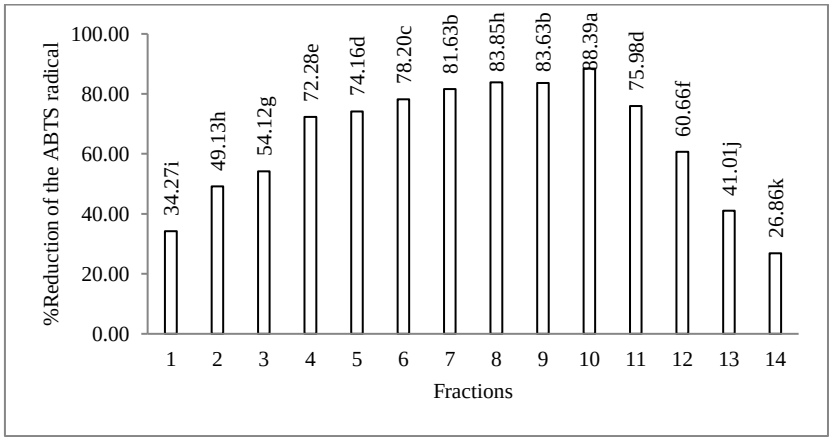

Fig. 4. Percent reduction of ABTS radical. Different letters indicate a significant difference (P≤0.05). Graph of the mean and standard error of three replicates.

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E. Iron-induced Lipid Peroxidation in Rat Brain Homogenate (TBARS)

Lipid peroxidation is a process of cellular damage, where the polyunsaturated lipids of the membranes are affected, forming products such as malondialdehyde (MDA). Fraction 10 is the one with the highest capacity to inhibit lipoperoxidation (Fig. 5), this is probably due to the higher concentration of anthocyanins since in the DPPH and ABTS tests it was the fraction with the highest antioxidant activity.

F. Stability of Anthocyanins in Relation to Temperature and pH

Anthocyanins are found in different chemical structures depending on the pH of the solution, so different shades are found at different pH values [15].

At pH 1, the flavilium cation (red color) is the predominant class and changes to purple and red colors. Between pH 2 and 4, quinoidal blue species are found. As the pH increases between 5 and 6, the carbinol pseudo base and a chalcone are distinguished, respectively [16]. As shown in Fig. 6 and Fig. 7.

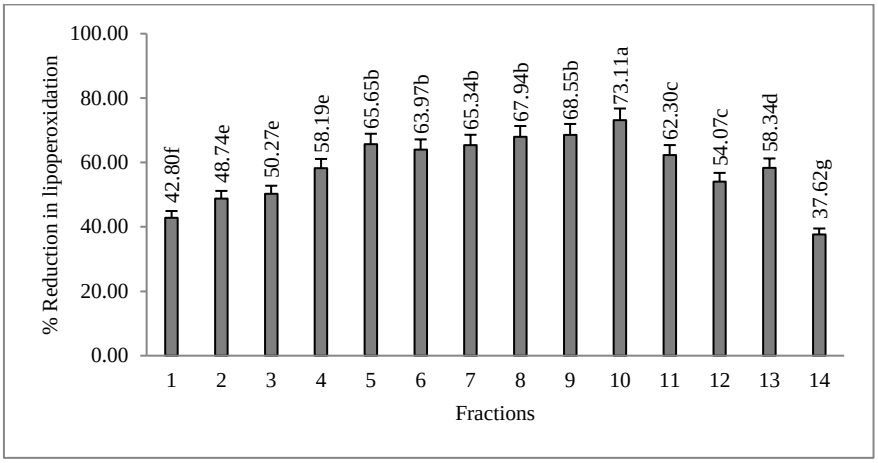

Fig. 5. Percentage inhibition of lipoperoxidation by the TBARS method from fraction 1 to 14. Different letters indicate a significant difference (P≤0.05). Mean and standard error of three replicates.

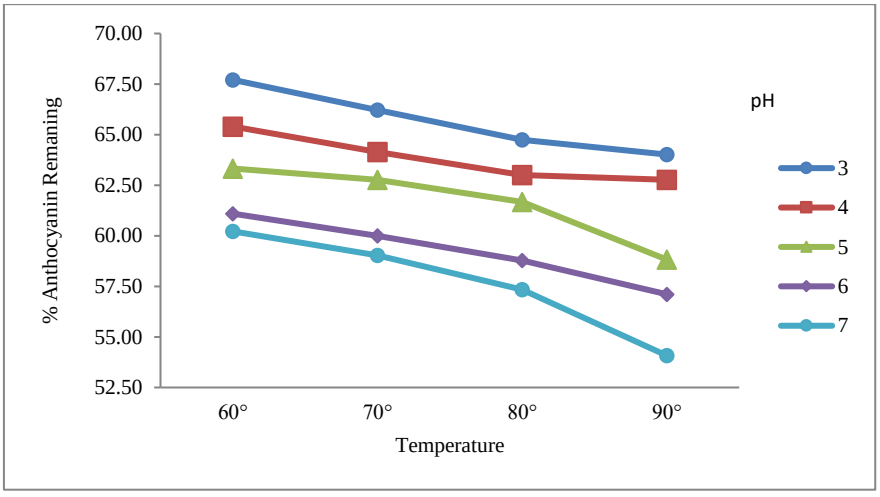

Fig. 6. Percentage of anthocyanin remaining against temperature (- 60°, -70°, -80°, -90°).

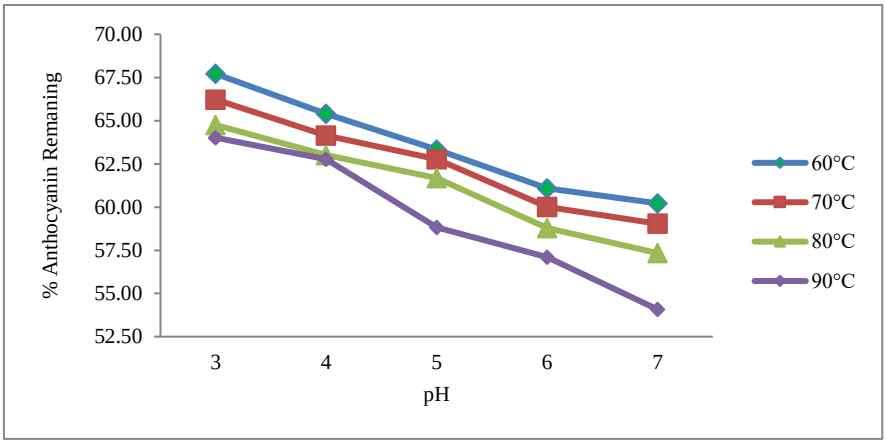

Fig. 7. Percentage of anthocyanin as a function of temperature at different pH (3,4,5,6 and 7).

G. Stability in Relation to Light

Light affects anthocyanins, although it is essential for biosynthesis and accelerates their degradation, they maintain their color in a better state when kept in the dark. Fig. 8 shows the percentage of anthocyanins remaining in relation to the days they were exposed to light.

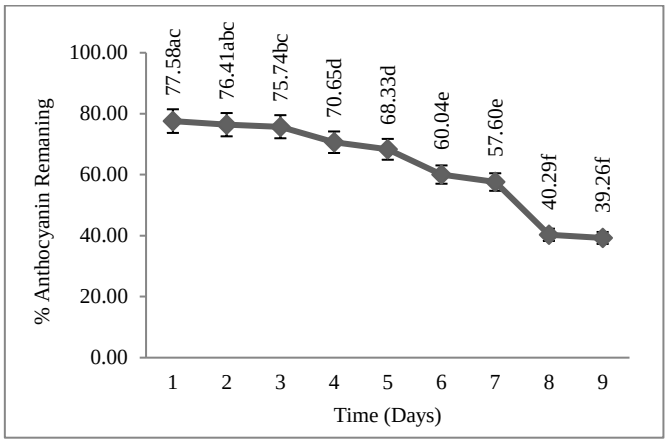

Fig. 8. Percentage of remaining anthocyanins as a function of days of exposure to light at pH 3. Different letters indicate a significant difference (P≤0.05). Plot of the mean and standard error of three replicates.

H. Sensory Evaluation of Anthocyanin in Commercial Plain Yoghurt

Sensory evaluation was carried out according to the methodology described above. The four parameters evaluated were: odor, color, flavor, and texture.

Based on the survey results, the attributes of smell, taste and texture were not found to have statistically significant differences (P≥0.05) among the untrained group of judges, while the color attribute was the one that resulted in significant difference (P≤0.05), with Danone yogurt pigmented with radish anthocyanin being preferred by the judges (Table II).

TABLE II: ANOVA AVERAGES OF THE ORGANOLEPTIC CHARACTERISTICS OF THE FOUR TREATMENTS. MEAN AND STANDARD ERROR OF THREE REPLICATES

	Alpura	Lala	Yoplait	Danone plus anthocyanin
Smell	25.29^{a}±1.42	25.00^{a}±1.34	23.98^{a}±1.60	25.73^{a}±1.86
Color	22.83^{a}±1.41	23.53^{a}±1.55	25.63^{ab}±1.29	28.01^{b}±1.45
Flavor	24.46^{a}±2.06	24.31^{a}±1.98	24.92^{a}±1.65	26.31^{a}±1.78
Texture	23.28^{a}±1.54	25.31^{a}±1.46	25.78^{a}±1.54	25.63^{a}±1.64

Different letters in the same row indicate significant statistical differences between treatments in a given attribute according to Tukey's test (P≤0.05) for the multiple comparisons test.

IV. DISCUSSION

In recent years, natural pigments have found considerable potential in the food industry as effective food colorants. Interest in anthocyanins as potential natural food colors has increased.

In Mexican society, the increase in the standard of living has involved a greater demand for food, but higher quality in relation to the quality of what is ingested, as well as a particular concern about food and its effect on health.

The epidermis of R. sativus has its coloration due to the high amount of anthocyanins: 17.36 mg/g Compared to samples of Brassica oleracea var. Capitata: 11.11 mg/g [17], Brassica rapa var Chinensis: 3.13 mg/g [18], Raphanus sativus L. var Niger: 6,820 mg/g [19] reported, probably due to stress by biotic and abiotic conditions such as: the presence of microorganisms, interaction with other plants; temperature, light, humidity, and soil [20].

Most vegetables of the Brassicaceae family are good sources of antioxidants, which ultimately benefit humans and promote plant health [21]. In general, radish contains higher amounts of anthocyanins, phenolic compounds, and antioxidant activity than white radish (Daikon) or, Raphanus sativus var longipinnatus.

Regarding the antioxidant activity of the 14 fractions extracted from the 695 g of macerated and purified epidermis, it can be observed that the results showed a high percentage of antioxidant activity in the ABTS and DPPH colorimetric tests, with a higher percentage compared to TBARS. The antioxidant activity is higher in fractions 7,8 and 9 due to a higher content of anthocyanins as suggested by thin-layer chromatography (Fig. 2), these compounds have a significant role in preventing lipid oxidation. Various anthocyanins have shown their efficacy in suppressing the development of lipoperoxidation of membrane phospholipids or linoleic acid, erythrocytes, or autooxidation of brain homogenates, as well as their efficacy in reducing low-density lipoprotein (LDL) oxidation in cultured macrophages in vitro and inhibiting the toxicity of LDL oxidases [22].

Research by [23] y [24] reported that the anthocyanins present in sativus radish are derived from Pelargonidin: Pelargonidin-3-sophoroside-5-glucoside acylated with hydroxycinnamic acids (p-coumaric, caffeic and/or ferulic acid). The major anthocyanins are Pelargonidin 3-O-[6-O-(E)-feruloyl-2-O-β-D-glucopyranosyl]-β-D-glucopyranoside]-5-O-(β-D- lucopyranoside) and Pelargonidin 3-O-[6-O-(E)-feruloyl-2-O-(6-(E)-cepheoyl- β-D-glucopyranosyl)- β-D-glucopyranoside]-5-O-(B-D-glucopyranoside).

The stability of anthocyanins in relation to temperature from different flowers and fruits has been studied by several researchers, proving that increasing temperature has a negative effect on anthocyanin content [25-27].

For the biosynthesis of anthocyanins, light is essential, but they also increase their degradation. Previous studies [28] showed that light accelerates some phytochemical changes in these pigments degrading them to the chalcone form and restricting their application as a food additive. The percentage of total anthocyanins decreased drastically from day 7 (Fig. 8) indicating that light affects the amount of anthocyanin and coloration.

In addition, the stability exposed to neon light and in the dark for 24 weeks has been investigated, showing that light causes a decrease in phenol and anthocyanin content during storage [29].

The data collected in the sensory test were subjected to an analysis of variance (ANOVA), using the level of preference as the response variable with a 95% confidence interval for the attributes of smell, color, flavor, and texture (Table II). To identify whether there were significant differences in the level of liking among these four attributes, it turned out that there was no difference in the level of liking among the attributes of the four samples. This means that the Danone®

sample pigmented with horseradish anthocyanin had a similar odor and flavor to the other samples and was superior in visual liking (color). The extracted anthocyanins are free of isothiocyanates since they did not present the pungent taste of radish. In addition, the color provided by the anthocyanins in the horseradish had a very homogeneous and attractive texture, similar to that of strawberry flavor, according to the participants of the panel of evaluators. Danone® plain yogurt pigmented with anthocyanin was very well accepted.

Regarding the estimated price of pigment extraction, this was $ 2 USD with a yield of 32 g of anthocyanins, and the artificial red dye no 40 $ 3.8 USD for a bottle of 28 g, this shows that horseradish anthocyanin can be used in the food industry with the benefit of producing a product free of synthetic dyes and with an extra portion of antioxidants that provide health benefits, thus yogurt added to these pigments can be used as a nutraceutical food.

V. Conclusion

Anthocyanins extracted from radish can function as a pigment in foods thanks to their bright red tone, in addition to being of natural origin and possessing antioxidant properties, they confer to the product the characteristic of a functional additive.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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