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Comparative study of anthocyanin extraction methods in *Dahlia pinnata* petals

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Summary

Anthocyanins are phenolic compounds responsible for the color of numerous plant sources. In literature, there is little information about the dahlia flower as a potential source of anthocyanins. This study aimed to develop a procedure for anthocyanins extraction from black dahlia petals using fresh and dehydrated material. A three-stages nested design was used to develop the methodology, 3 solid-liquid-ratios and 6 dissolvents nested in 3 methods. The highest yields were obtained with the homogenization assisted maceration technique, citric acid solvent (2, 4, 6%), and a ratio of 1:30 with dry petals. The results of this study show the opportunity to obtain a high anthocyanin content from black dahlia and open the possibility to use it as another important source of this pigment.

Keywords: Dahlia, anthocyanins, sonication, maceration, homogenization, extraction.

Introduction

The colorants used in the food industry are generally of synthetic origin and are used to improve visual perception or when the natural color has been affected during processing, storage, or distribution (Coultate and Blackburn, 2018). However, concerns about health and restriction in the use of synthetic colorants in several countries have led to a tendency to replace artificial colorants with natural pigments (McGill, 2009).

Anthocyanins are a representative group of natural pigments; since of their wide distribution, water solubility, and health benefits such as antioxidant, anti-cancer, anti-diabetic, and visual health improvement effects (Khoo et al., 2017). In addition, they have a wide range of colors ranging from red, blue, purple, magenta, and orange. This latter feature added to their health benefits has allowed its application in the food industry and led to the constant search for sources of this pigment such as grape skin, blueberry, cranberry, raspberry, blackberry, radish, cherry, rosella, purple cabbage, among others (Leong et al., 2018). Dahlia flower with red, purple, and black shades owes its hue to the presence of anthocyanins (Lara-Cortés et al., 2014; Deguchi et al., 2016)

This flower is native to Mexico, belongs to the Asteraceae family, and is part of the Heliantheae tribe, which the Aztecs called *Cocoxochitl* because of its hollow stem. In pre-Hispanic times it had a dominant culinary and decorative use. In addition, its petals were used to extract pigment and color cotton (Luna Monterrojo, 2008; Lara-Cortés et al., 2014). In literature, information about dahlia flower as a source of anthocyanins for food application is scarce, so the study of pigment content is a fundamental factor in defining its potential; therefore, it is essential to develop extraction processes that help to know the conditions that lead to its optimization. In most cases, extraction of anthocyanins for their application in food is carried out with solvents such as water and ethanol, which are usually acidified with weak acids such as acetic and citric (Belwal et al., 2018).

The subject of this work was *Dahlia pinnata*, and the objective was to perform a comparative study of anthocyanin extractions in fresh and dried petals, so three different extraction techniques were used with variations in solvents and solid-liquid ratio.

Materials and methods

Dahlia flowers were collected in the municipality of Huamantla, located in the eastern part of the state of Tlaxcala, Mexico (coordinates: 19.3420758, -97.91872470000001). The dahlia petals were manually removed from flowers, 13 kg of this material were obtained; 3 kg were used for fresh experiments and the remainder was dried at a temperature of 30 °C in a drying oven (SEV® s/n), up to a constant weight. Dehydrated petals were vacuum-packed and stored in darkness at room temperature until use.

Extraction procedure for anthocyanins

Since dahlia blooming is annual, it is considered important to study in three extraction conditions of fresh and dehydrated material. The extraction was performed by maceration which involves the simple diffusion of the pigment towards the liquid phase, using three solid ratios (fresh or dehydrated), with six different types of solvent (liquid phase) and for the extraction three conditions of the solid material (simple maceration, assisted maceration with homogenization and assisted maceration with sonication). In all of them, the ratio (S/L) or solid/solvent was 1:10, 1:20 and 1:30, using distilled water, acidified at 2, 4 and 6% with citric acid, 2% ethanol, and 2% acetic acid. After each extraction was finished, the liquid extract was separated from the solid residue through a line cloth filter and subsequently with 0.25 µm Whatman member filter twice. All experiments were performed in triplicate using amber material and in the absence of illumination.

Simple maceration

This conventional extraction was carried out in a 100 mL flask for 24 hours at 25 ºC. Dahlia petals (1.5 g) were mixed with the different solvents to give the ratios 1:10, 1:20 and 1:30. This method was called maceration.

Homogenization assisted maceration

About 1.5 g of plant material was extracted with the different solvents at the indicated rates, by mechanical way. This method was called homogenization.

Sonication assisted maceration

An ultrasound-assisted extraction process was carried out with 1.5 g of fresh and/or dry material. Later, different solvents were added

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to give the respective mass liquid ratios. Samples were treated for 60 minutes using a Branson 3800H ultrasound bath (110 W, 40 kHz) at 25 ºC. This experiment was called sonication.

Total anthocyanin content

The total monomeric anthocyanin was quantified through the differential pH method (HUTABARAT et al., 2019). Values were expressed in mg of cyanidin-3-glucoside and calculated as follows:

$$\text{Anthocyanin Extraction Yield (mg/100 g)} = \frac{Abs \times MW \times DF \times V \times 1000}{(\varepsilon \times L \times m)} * 100$$

Where: *MW*: Molecular weight of cyanidin-3-glucoside (449.2 g/mol), *DF*: Extract dilution factor, *V* = Volume of extract (L), 1000 = Conversion factor from g to mg, ε = molar extinction coefficient of cyanidin-3-glucoside (26900 L/mol.cm) *L* = Length of the cuvette (cm), *m* = weight of the sample (g) in dried basis. Finally, the results were expressed as mg of cyanidin-3-glucoside per 100 g. In any case, the result of the pigment has been adjusted to the solid content in the material.

Scanning electron microscope (SEM)

Material observations before and after extraction were performed by scanning electron microscopy; for this purpose, samples of dry petals with the highest and lowest anthocyanin yield obtained were selected. Therefore, the micrographs were: homogenization with 2% citric acid, simple maceration with 2% acetic acid, sonication with 4% citric acid, simple maceration with 2% acetic acid, and maceration with 2% citric acid, all of them with the solid-liquid ratio at 1:30, finally the control it was the petal with no treatment. After extraction, the samples were dried to constant weight and stored in vacuum-sealed flasks. Subsequently, they were gold-plated in a Denton Vacuum Desk V equipment, and the images were obtained through the JSM 66101V electron microscope.

Statistical analysis and graphs

The experimental design was a nested design type (Fig. 1), in which the treatments were: homogenization, maceration, and sonication. The factors were the solid-liquid ratio (levels: 1:10, 1:20, and 1:30) and the solvent (levels: water, citric acid 2%, 4%, and 6%, 2% ethanol, and 2% acetic acid). The statistical software Minitab 16 (Minitab Inc.) was used to perform the variance analysis (ANOVA) followed by Tukey's multiple comparison test. The confidence level used for the variance analysis was 95%. Extraction graphics were performed with GraphPad Prism version 8.4.0 program.

Results and discussion

Fresh material

Tab. 1 summarizes the reported anthocyanin content in mg/100 g (dry base) extracted under different extraction conditions. The method that influenced obtaining the highest yields was maceration followed by grinding and sonication. It was not possible to extract the pigment in maceration using water as a solvent, because immediately extract turned brown showing its oxidation. The same phenomenon happened with sonication using water, it is deduced that prolonged time could induce pigment degradation. Previous studies indicate that the extraction of anthocyanins using ultrasound leads to oxidation reactions and it has also been reported that prolonged sonication times influence the degradation of phenolic compounds such as anthocyanins (MANE et al., 2015; DAILEY and VUONG, 2015).

Fig. 2 shows the solvent that contributed to high yields (6.63-13.67 mg/100 g) was generally 2% acetic acid ($p < 0.05$), followed by acidified water with 6% citric acid at intervals of (1.50-8.23 mg/100 g), unlike distilled water, which was the solvent with the lowest yields (1.20-2.42 mg/100 g) and in some cases not detected. It is therefore clear that an acid medium is needed to provide stability to anthocyanins. Acidified solvents are more efficient in extracting anthocyanins than those that are neutral, since at different pH values different anthocyanin structures predominate, for example, at pH values below 4 the predominant form is the flavillium cation, whose structure is more stable than the others (quinoidal base, carbinol pseudo base, and chalcone (AZWANIDA, 2015; NGAMWONGLUMLERT et al., 2017).

The combination of factors that led to obtaining the highest yield corresponds to maceration using 2% acetic acid with the ratio of 1:30 (13.67-5.85 mg/100 g of anthocyanin), unlike the lower yields that were tested with sonication, using water with 2% citric acid with the same solid-liquid ratio (0.58- 0.25 mg/100 g anthocyanin).

Finally, statistical analysis will conclude that the different solid-liquid ratios proposed in this work (1:10, 1:20, and 1:30); do not affect the extraction when petals are fresh.

Dried material

The homogenization method showed the highest extraction yields, followed by maceration and sonication in dehydrated dahlia petals (Tab. 2). These results can be attributed to the fact that homogenization, compared to maceration, leads to cell lysis through shear forces that release the pigment to the medium, resulting in a faster diffusion process. Another factor to consider is extraction time, which, in the case of maceration ends when a balance is reached between the concentration of metabolites in the extract and petals, so it can take hours and even days to obtain higher yields. These results co-

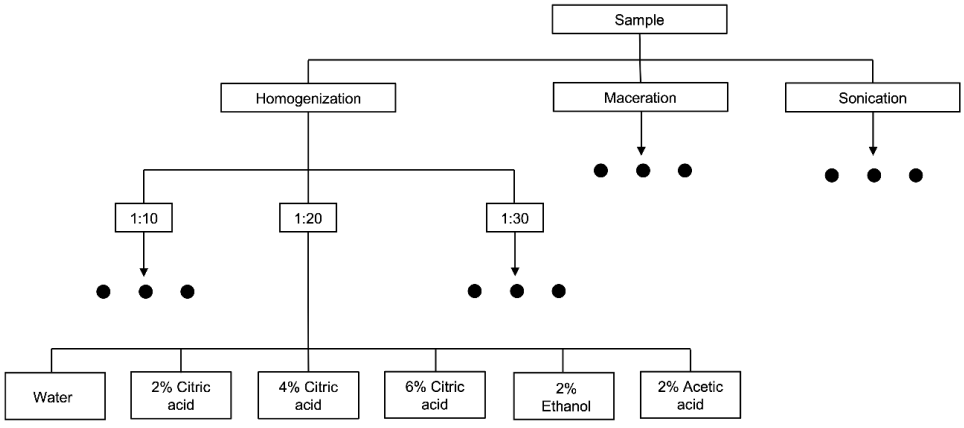

Fig. 1: Experimental design of extraction treatments
The black-spot shapes indicate repeated experiment

Tab. 1: Anthocyanin yields in fresh dahlia petal extracts

	Anthocyanins mg/100 g					
			Disolvent			
Ratio	**Water**	**2% Citric acid**	**4% Citric acid**	**6% Citric acid**	**2% Etanol**	**2% Acetic acid**
			Homogenization			
R 1:10	n.d.	6.30 ± 0.24	6.03 ± 0.39	8.23 ± 1.03	3.21 ± 0.43	*7.95 ± 0.88
R 1:20	n.d.	6.31 ± 1.88	5.22 ± 0.78	5.73 ± 0.32	3.09 ± 0.26	*10.78 ± 0.53
R: 1:30	n.d.	5.24 ± 1.07	5.26 ± 1.33	6.41 ± 0.44	2.88 ± 0.00	*6.53 ± 0.56
			Maceration			
R 1:10	1.20 ± 0.19	4.87 ± 0.43	2.21 ± 0.37	4.38 ± 0.06	4.13 ± 0.54	*8.97 ± 0.76
R 1:20	2.34 ± 0.44	4.87 ± 0.36	4.29 ± 0.42	3.76 ± 0.22	4.63 ± 0.84	*10.78 ± 0.04
R: 1:30	2.42 ± 0.18	5.05 ± 028	5.03 ± 0.56	4.61 ± 0.90	3.99 ± 0.22	*13.67 ± 5.85
			Sonication			
R 1:10	n.d	0.96 ± 0.10	0.94 ± 0.15	1.25 ± 0.30	5.65 ± 0.43	*6.63 ± 0.80
R 1:20	n.d.	0.70 ± 0.46	0.97 ± 0.21	5.20 ± 0.34	5.20 ± 0.39	*8.29 ± 0.90
R: 1:30	n.d.	0.58 ± 0.25	0.97 ± 0.47	1.50 ± 0.66	4.55 ± 0.64	*7.83 ± 1.32

The * symbol indicates significant differences (p <0.05)

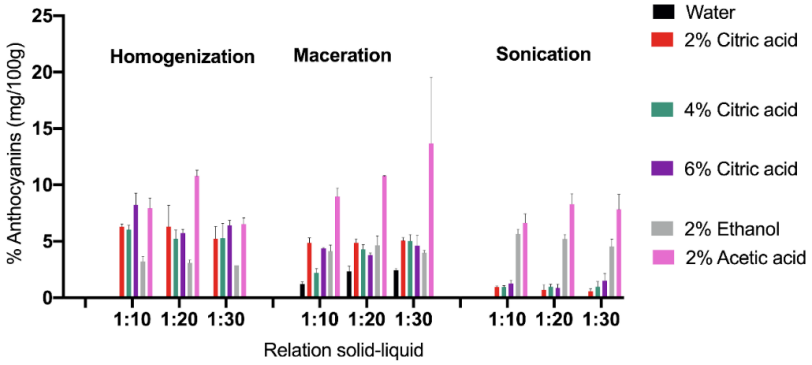

Fig. 2: Anthocyanin yields in fresh dahlia petal extracts

Tab. 2: Anthocyanin yields in dried dahlia petal extracts

	Anthocyanins mg/100 g					
Ratio	**Water**	**2% Citric acid**	**4% Citric acid**	**6% Citric acid**	**2% Ethanol**	**2% Acetic acid**
			Homogenization			
R 1:10	12.3 ± 1.4	*27.07 ± 9.40	*26.87 ± 4.04	*33.19 ± 1.86	1.23 ± 0.43	4.86 ± 1.66
R 1:20	16.63 ± 2.10	*35.21 ± 3.88	*38.27 ± 4.66	*29.50 ± 0.64	1.22 ± 0.21	2.64 ± 1.27
R: 1:30	16.68 ± 2.08	*36.53 ± 2.37	*24.42 ± 15.04	*30.48 ± 3.45	1.01 ± 0.19	2.17 ± 0.42
			Maceration			
R 1:10	3.39 ± 0.65	6.39 ± 0.33	3.60 ± 0.88	7.33 ± 0.73	0.87 ± 0.35	4.00 ± 0.34
R 1:20	4.90 ± 0.28	12.58 ± 3.34	12.26 ± 1.95	11.29 ± 1.06	1.06 ± 0.15	5.33 ± 0.61
R: 1:30	6.91 ± 0.56	19.79 ± 3.56	17.87 ± 1.96	26.86 ± 8.40	1.02 ± 0.17	4.49 ± 0.99
			Sonication			
R 1:10	2.93 ± 0.80	1.81 ± 0.22	3.73 ± 1.48	4.46 ± 0.54	1.05 ± 0.17	3.01 ± 0.17
R 1:20	3.83 ± 0.79	0.47 ± 0.01	4.76 ± 2.22	1.36 ± 1.11	1.36 ± 0.24	3.27 ± 0.38
R: 1:30	6.18 ± 1.49	0.07 ± 0.01	12.94 ± 5.67	10.46 ± 3.20	1.77 ± 0.19	2.40 ± 0.91

The * symbol indicates significant differences (p <0.05)

incide with other research published and carried out on other biological materials (SARKER et al., 2006; ROSTAGNO and PRADO, 2013; AZWANIDA, 2015).

On the other hand, the difference that led to homogenization is superior to sonication (although both are methods of cellular disruption) is due firstly to the fact that homogenization is less aggressive and secondly to the fact that the time spent in sonication was probably very long.

Solvents with higher yields were citric acid at different concentrations, with no significant differences (p <0.05), followed by water, 2% ethanol, and 2% acetic acid. As mentioned above, acidified systems promote the stability of anthocyanins and it is also known that an acidic pH promotes the cleavage of phenols bound to proteins and carbohydrate polymers since phenols are protonated at low pH values

and adopt a hydrophobic nature that allows them to penetrate the micelles with a surfactant effect (Chaves et al., 2020).

Regarding the solid-liquid relationships raised, there were significant differences ($p < 0.05$), being the 1:30 relationship the one that contributed to obtaining the highest yields in the three different methods. Fig. 3 shows the values obtained. As noted, homogenization along with acidified water were the factors that led to higher extraction yields, however, no significant differences($p < 0.05$) were found between the use of citric acid at 2, 4, and 6%, therefore the use of the 2% is recommended since a smaller amount of the acidulant is required. Finally, the solid/liquid ratio 1:30 showed a yield interval between 24.42 and 35.53 mg/100 g of pigment.

In summary, extraction yields in dry petals were higher than fresh material, with a content of up to 35.53 mg/100 g, compared to the fresh samples where the maximum yields were 13.67 mg/100 g.

SEM analyses

Fig. 4 shows SEM images of cellular morphological changes in the epidermis caused by maceration, sonication and homogenization methods on dry petals. The control (Fig. 4a) is a surface structure of cells of the papillose type, freely arranged and with large intercellular spaces. In the maceration process with 2% citric acid (Fig. 4b), moderate changes in morphology were observed, some agglomerations of apparently empty cavities indicate that there was an extraction process. In the sample extracted with homogenization in the presence of acetic acid (Fig. 4c), it is observed that the structure of the cones is preserved, only more agglomerates are observed, so the extraction could not be as efficient. The homogenization carried out with 2% citric acid (Fig. 4d) led to the petal having notable morphological changes; as the formation of cavities due to transferring the cellular content to the solvent and slight fragmentation of the structures.

This fragmentation increases the surface, which increases the mass transfer and extraction efficiency. For the petals with a sonication process in 2% citric acid (Fig. 4e) and with 2% acetic acid (Fig. 4f), more drastic morphological changes are shown, presenting a fragmentation of structures and erosion processes, probably caused by the implosion of bubble cavities on the surface of the petals, causing a release of cellular contents to the extraction medium (Chemat et al., 2017; Bailes and Glover, 2018).

Conclusions

Based on a comparative study of extraction methods in dried dahlia petals, the factors that led to achieving the highest yields of 24 to 38 mg/100 g were through homogenization using water acidified with citric acid 2-6% with a ratio of 1:30. For fresh petal yields, the

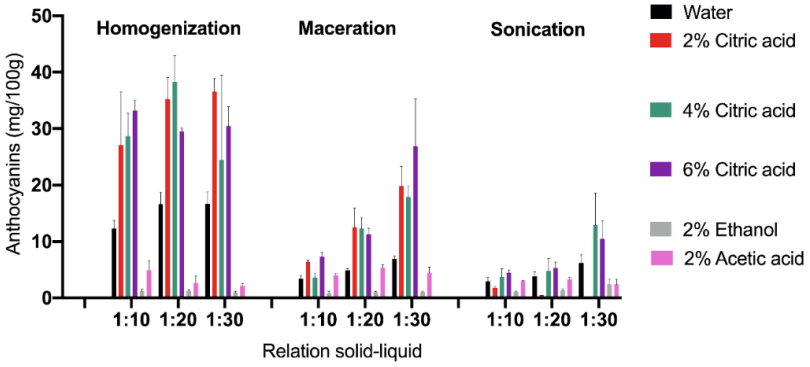

Fig. 3: Anthocyanin yields in dried dahlia petal extracts

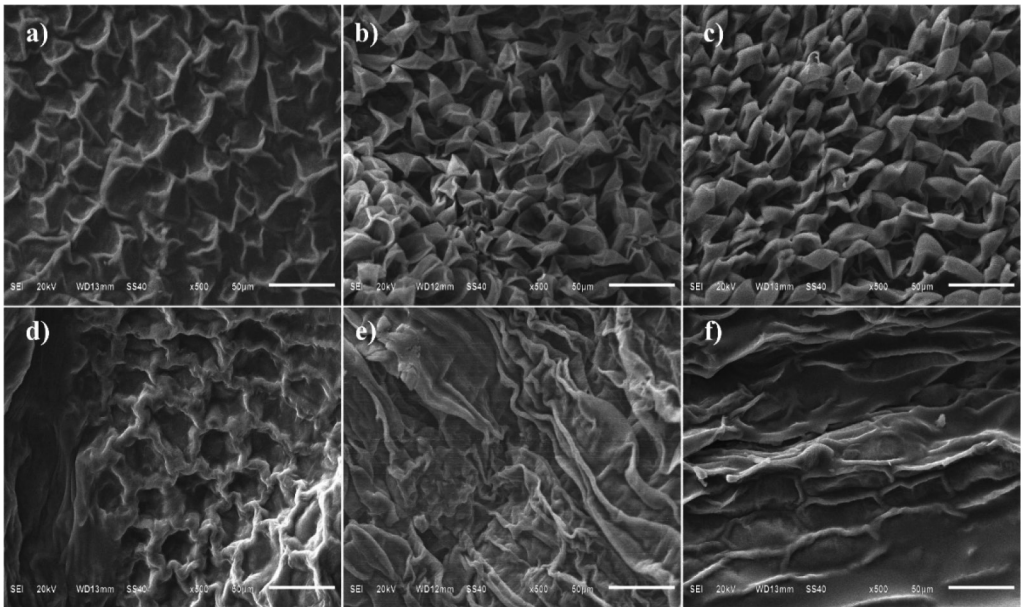

Fig. 4: **a)** control. **b)** maceration with 2% citric acid. **c)** homogenization with 2% acetic acid. **d)** homogenization with 2% citric acid. **e)** sonication with 4% citric acid. **f)** sonication with 2% acetic acid**.**

highest values were obtained by maceration with 2% acetic acid in a solid-liquid ratio of 1:30. The images of the SEM showed that there were changes in the cellular morphology of the epidermis of the dried petals with every method of extraction; homogenization, maceration, and sonication. The most notable change occurred with sonication since fragmentations were observed in the cellular structure of the epidermis. Finally, due to the results obtained show a high content of anthocyanins, it is concluded that this flower has potential for application in food, however, it is suggested to carry out safety tests as well as to evaluate the stability of the pigment.

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Conflicts of interest
No potential conflict of interest was reported by the authors.

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