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New acylated cyanidin glycosides extracted from underutilized potential sources: enzymatic synthesis, antioxidant activity and thermostability.

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ABSTRACT

Interest in anthocyanins has increased remarkably in recent decades, although their wider application has been hampered by instability problems. Thus, this study aimed at developing a strategy to gain access to more stable anthocyanins via enzymatic esterification. For that purpose, three cyanidin derivatives were obtained from underutilized, but easily accessible sources, and their total anthocyanin content was quantified. The purity of cyanidins obtained ranged from 40 % to 88 % depending on their source. Subsequently, the critical enzymatic reaction conditions were established, and the best results were found using *tert*-butanol as a solvent, 20 g/L of lipase B from *Candida Antarctica,* and vinyl cinnamate as acyl donor at ratio 250:1 (acyl donor to anthocyanin). Finally, five new acylated anthocyanin derivatives were synthesized with improved antioxidant activity and thermostability, in comparison to the cyanidin-3-glucoside, which is an advantageous feature for industrial applications.

Keywords: lipases, enzymatic esterification, dihydrocinnamic acids, acylated anthocyanin.

1. Introduction

Anthocyanins are water-soluble pigments present in fruits, leaves, flowers, and roots. Due to their antioxidant activity and biological properties, the research on anthocyanin applications has attracted a considerable interest in food, nutraceutical, cosmetic, and pharmaceutical industries (Nunez & Magnuson, 2014). For example, it was proved that they played a role in obesity prevention and cardiovascular protection. Nevertheless, the use of anthocyanins in the industry is limited because of their instability (Yan, Li, Zhang, Liu, Ou, & Zeng, 2016) and availability (Cruz, Fernandes, Guimarães, de Freitas, & Mateus, 2016).

Anthocyanins are known to be unstable when subjected to the changes, such as pH, temperature, exposure to light, and are also affected by the presence of oxygen, enzymes and metals. However, it is well documented that acylated anthocyanins show improved stability in comparison to non-acylated ones. It has been demonstrated that acylation with aromatic acids is especially efficient (Li *et al*., 2013). The observed higher stability is explained by the intramolecular copigmentation caused by π-π interactions (Trouillas, Sancho-García, De Freitas, Gierschner, Otyepka, & Dangles, 2016). Therefore, it is important to gain access to those types of anthocyanin derivatives from natural sources,(Gras, Bogner, Carle, & Schweiggert, 2016), or by esterification of glycosylated anthocyanins. Esterification can be achieved chemically or enzymatically. Chemical esterification presents certain disadvantages. It usually involves addition of bases that degrade anthocyanins, and is characterized by poor selectivity, that is, it takes place both at glycoside and aglycone residues (Grajeda-Iglesias, Salas, Barouh, Baréa, & Figueroa-Espinoza, 2017). On the contrary, enzyme-catalyzed esterification is performed at soft reaction conditions and with high selectivity. In previous works, enzymatic esterification of anthocyanins was carried out using 10 % DMSO in acetone (Sari, Setiawan, & Siswoyo, 2015),(Cruz, Benohoud, Rayner, Mateus, Freitas & Blackburn, 2018), in pyridine (Yan *et al.*, 2016) or in 2-methyl-2-butanol (Cruz, Guimarães, Araújo, Évora, de Freitas & Mateus, 2017) as reaction solvents. However, those studies lacked the solvent assessment and systematic evaluation of factors distinct from flavonoid esterification, such as water activity, type of solvent, the ratio between acyl donor: acyl acceptor, temperature, type and concentration of acyl acceptor, acyl donor and enzyme (Koskien & Klibanov, 1996). Factors listed above comprise the critical conditions to perform esterification in organic media, which is often limited by the solubility of the substrates. For that reason, in previous studies a range of solvents for flavonoid esterification were tested (hexane, acetone,

acetonitrile, pyridine and mixture of them) with outcome greatly depending on the substrate properties (Chebil, Humeau, Falcimaigne, Engasser, & Ghoul, 2006).

Another drawback related to anthocyanin esterification is the reaction yield. Owning to the lack of commercial sources of these compounds, the inevitable first step involves extraction and purification of anthocyanins, so that esterification studies could be carried out at all. Although these pigments are distributed throughout nature, it is necessary to find their accessible sources with a simple profile and high anthocyanins concentration, which would facilitate more specific esterification studies. Different authors have reported the extraction of malvidin-3-glucoside from grapes; cyanidin-3-glucoside from jaboticaba, purple rice, and raspberry to prepare their corresponding esters, the type of donor acyl that has been evaluated is mainly fatty acids which are more liposoluble products, however, esterification with aromatic acids has been evaluated slightly. In those studies, only four plant sources were evaluated, and two different anthocyanins were obtained. However, given the great diversity of anthocyanins and their plant sources, it is necessary to expand the access and characterize new structures belonging to these biologically active pigments. In Mexico, thanks to its biodiversity, a vast number of plants can be considered as a new and accessible anthocyanin sources, such as purple corn *(Zea mays*) (Deineka, Sidorov, & Deineka, 2016), tiliapo (*Sideroxylon palmeri*), plum (*Prunus domestica*)(Usenik, Stampar, & Veberic, 2009), trueno (*Ligustrum japonicum*), and bottlebrush flower (*Callistemon citrinus*)(Goyal, Jain, Jain, & Sharma, 2012). All of them possess high anthocyanin content and are widely distributed in Mexico. For example, although the purple corn is used as source of anthocyanins, their use is not taken advantage in the whole plant, for example purple corn husk is a by-product of purple corn production which is used to prepare traditional food as atole (Chung-Ying *et al*., 2008); trueno and *Callistemon* trees grow abundantly in the streets of Mexico City (Aldama & Grabinsky, 1994) and additionally are used in traditional medicine since they act not only as antioxidant, antimutagenic, cytoprotective, and immunosuppressive agents (trueno)(Ngo, Lee, Nguyen, Kim, Woo & Min, 2017), but also show antibacterial activity (bottlebrush leaves and flowers)(Cock, 2012); tiliapo is utilized as food in its immature and mature state (González-Soberanis & Casas, 2004) though the knowledge of its chemical composition is limited; plum is a readily available fruit known for its remarkable antioxidant properties (Cock, 2012) together with its antibacterial and anticancer activities (El-Beltagi, El-Ansary, Mostafa, Kamel, & Safwat, 2018).

Therefore, the main goal of this work was to obtain novel acylated cyanidin glycoside derivatives through enzymatic synthesis and evaluate their antioxidant activity and thermostability. In order to improve the enzymatic reaction yields, the effects of solvent, enzyme, acyl donor, and glycosyl moiety of the cyanidin were studied.

2. Materials and methods

2.1. Materials

Vinyl cinnamate, cinnamic acid, solvents and commercially available immobilized lipase from *Candida antarctica* (> 5000 U/g, recombinant, expressed in *Aspergillus niger*), Amberlite XAD-7HP, Sephadex LH-20, trifluoroacetic acid were purchased from Sigma-Aldrich. Dihydrocinnamic acid, dihydroferulic acid, and dihydrosinapic acid were obtained by hydrogenation of cinnamic, ferulic and sinapic acid using a Parr hydrogenator and Pd/C as catalyst.

2.1.1. Plant material obtention.

In the case of the tiliapo and the plum, only the husk of the ripe fruit was used, unlike the thunder in which the whole fruit was used because this fruit is too small to remove the husk, in the case of corn, the leaves covering the fruit (husk) were used, and finally only the colorful part of the flower was used.

One of the anthocyanins of the red cabbage was used as anthocyanin control in the antioxidant activity. The red cabbage were extracted purified until obtained the a natural anthocyanin isolated from red cabbage was used, which structure tentatively corresponds to cyanidin-3-sinapic-diglucoside-5-glucose (Cy-3-sin-diglc-5glc)(Mizgier, Kucharska, Sokół- Łętowska, Kolniak-Ostek, & Kidon, 2016)

2.2. Determination of total anthocyanin content (TAC)

The monomeric anthocyanin pigment content in the extracts from the five different plant sources was determined using the pH differential method, as previously described(Giusti & Wrolstad, 2001). TAC was determined spectrophotometrically using the extinction coefficient (Jing & Giusti, 2007) of 26900 L cm^{-1} mg^{-1} and a molecular weight of 449.2 g mol^{-1}, and was expressed as equivalents of cyanidin-3-glucoside per gram of dry weight of the sample. The buffer system used in the assay was 0.025 M potassium chloride at pH 1.0 and 0.4 M sodium acetate at pH 4.5. In brief, 200 µL of the extract were mixed separately with 1800 µL of potassium chloride and sodium acetate buffer, and the absorbance at 510 and 700 nm was measured using a visible-UV spectrometer (GBC-CINTRAL). The samples were tested in triplicate.

112 *2.3. Extraction of cyanidin glycosides*

113 Anthocyanins from different plant sources were extracted following the same method, though samples differed
114 on their initial weight. Cyanidin-3-glucoside was obtained from 200 g of tiliapo husk (*Syderoxilon palmeri*) and
115 500 g of corn husk (*Zea mays)*; cyanidin-3-rutinoside was obtained from 20 g of plum husk (*Prunus domestica*)
116 and 50 g of trueno fruit (*Ligustrum japonicum*); and cyanidin-3,5-diglucoside was obtained from 10 g bottlebrush
117 flower (*Callistemon citrinus).*

118 For the extraction process, test samples were suspended in the solvent mixture of methanol: water: formic acid
119 with volume ratio of 80:19:1, and then were sonicated for 20 min. Next, samples were filtered off and
120 concentrated under reduced pressure. Additionally, washings with hexane and ethyl acetate were carried out to
121 extract non-anthocyanin organic compounds from the sample. Following that, the sample was loaded to the
122 aqueous phase on Amberlite XAD-7HP resin was used to absorbed the anthocyanins in a ration of 1:10 of
123 sample in weight and resin. Subsequent washings with water were performed until colorless eluent was
124 obtained, the amount of water used was about from 1 L per 1 gram of material, however this value is variable
125 and depend of each sample. Then, anthocyanins were removed from Amberlite resins with methanol in a ratio
126 1:5 and this process was repeated three time and the layers was mixed, and excess of the solvent was
127 evaporated under reduced pressure till obtained a dark solid.

128 *2.4. Purification of anthocyanins extracts*

129 The crude extracts from different food sources were dissolved in 0.1 % TFA in methanol. Sephadex LH-20
130 column chromatography was performed using 0.1 % TFA aqueous solution as the mobile phase to separate the
131 anthocyanins. Anthocyanins fractions were concentrated under reduced pressure until dark solids were
132 obtained. Resulting anthocyanin trifluorate salts were weighted. All anthocyanins were identified by HPLC-MS.
133 Additionally, the anthocyanins extracted were confirmed by ^{1}H-NMR and ^{13}C-NMR.

134 *2.5. Evaluation of enzymatic esterification conditions*

135 Esterification of anthocyanins was achieved by enzymatic transformation using lipase B from *Candida antarctica*.
136 The reaction required a high-purity anthocyanin as acyl acceptor and a carbonyl group as acyl donor. In this
137 study, sequential experiments were performed to identify critical conditions. All reactions were carried out in a 20
138 mL vial, and the working volume was 3 mL. Temperature (60 ºC), agitation rate (300 rpm), and incubation time
139 (48 h) were maintained constant throughout the optimization process.

Effect of solvent: three organic solvents were tested as reaction media for anthocyanin and vinyl cinnamate substrates in lipase-catalyzed esterification. Solvents tested were dry pyridine, *tert*-butanol, and 2-methyl-2-butanol. 1 mL of a test solvent was added to 5 mg of cyanidin-3-glucoside, and the solution was left stirring for 24, 48 and 72 h at 300 rpm and 60 ºC. After that time, the substrate concentrations were measured by HPLC at 520 nm taking methanol as 100% solubility.

Effect of enzyme: three lipases on immobilized support were evaluated: lipase B from *Candida antarctica* (≥ 5000 U/g, Sigma-Aldrich), C-LECTA (2612 U/g for PNPB at pH 7, 25 °C), and lipase from *Candida cylindraceae* (1257 U/g for PNPB at pH 7, 25 °C). 5 mg of cyanidin-3-glucoside and 500 mL of vinyl cinnamate were transferred to the reaction vials containing 15 mg, 30 mg, and 60 mg of the test enzyme. Reactions were carried out at 60 °C and 300 rpm for 48 hours. After that time, the reaction was stopped by filtration, and the solvent was evaporated under reduced pressure. Next, vinyl cinnamate was removed with hexane, and the anthocyanin and respective ester were recovered with acidified water. Reaction products were analyzed by HPLC.

Effect of acyl donor: Three derivatives, namely vinyl cinnamate, dihydrocinnamic acid, and cinnamic acid were examined as acyl donors at the following concentrations: 50, 100, and 250 equivalents.

Effect of glycosyl moiety. Finally, cyanidin-3-glucoside, cyanidin-3-rutinoside, and cyanidin-3,5-diglucoside were used in esterification reactions to investigate the effect of the glycosyl moiety. The reaction conditions were as follows: 20 g/L of the enzyme, 250 equivalents of acyl donor, 100 g/L of molecular sieves 4Å in *tert*-butanol at 60 ºC, 300 rpm and for 48 h.

2.6. Purification of anthocyanins esters

The monitoring of the reaction was done by HPLC, when it was observed that the concentration of the product began to decrease, and the reaction stopped. The esterification reaction was stopped when the reaction was filtered. The solvent was concentrated in a rotavapor and the acidified water was dissolved with HCl to later wash with ethyl acetate and remove the excess from the acyl donor. The aqueous phase was set aside and led to dryness, this extract contained cyanidin that did not react with its esters, to separate these compounds was used a column silica (20 cm x 2 cm) using as hexane eluent to remove the excess pyridine, and then with ethyl acetate: methanol: formic acid (80:10:10). The analyses of thermostability and antioxidant activity were performed with the purified products.

The conversion yield was calculated by HPLC. Once the reaction product was identified by HPLC-MS, the concentration was calculated using the standard curve of the cyanidin-3-glucoside. The calculation of the yield was made by dividing the obtained value by the expected value per 100, the expected value being the total conversion of the cyanidin-3-glucoside.

2.7. HPLC / HPLC-MS

The reaction mixture was evaporated under reduced pressure and dissolved in acidified water. Next, extraction with hexane was performed to remove the excess of acyl donor. The aqueous phase was further concentrated under reduced pressure and analyzed by HPLC (Waters 2707) and HPLC-MS (Agilent 1260 Infinity Binary) using the ODS-Hypersil Gold column (250 mm x 5.4 mm x 5 μm). Detection was performed at 280 nm and 520 nm (dual UV-VIS detector, Waters 2847). The binary mobile phase consisted of solvent (A) water/acetic acid 94:6 (v:v) and (B) acetonitrile/acetic acid 94:6 (v:v); gradient elution was applied at flow rate of 0.6 mL/min: 0-25 min, from 4 % to 40 % B; 25-35 min from 40 % to 80 % B; 35-45 min from 80 % to 5 % B; 45-52 min, 5 % B (Yan *et al.*, 2016) and the injection volume was 10 μL.

Mass spectrometry (Agilent MS QQQ) in positive ion mode was selected to record the full mass spectra (*m/z* 50-1650) on an electrospray ionization ion-trap mass spectrometer (ESI-MS). The pressure of nebulizer gas (N_2) was set at 40 psi and a flow rate at 10 L/min. The capillary temperature was controlled at 300 ºC, and capillary voltage was maintained at 4 kV.

2.8 NMR

^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectra were recorded in CD_3OD:CF_3COOD (1 %) using an Agilent DD2 600 with One NMR probe and TMS as an internal standard. ^{1}H chemical shifts were assigned using 2D NMR (COSY), and ^{13}C chemical shifts were assigned using HSQC and HMBC. Obtained data were analyzed and compared with the available literature on structural analysis of cyanidin-3-glucoside (Oliveira, Fernandes, & de Freitas, 2016), cyanidin-3-rutinoside (Qin, Li, Niu, Ding, Zhang & Shang, 2010), and cyanidin-3,5-diglucoside, moreover the recopilation of the different anthocyanins NMR spectrum were use as comparison. (Andersen & Fossen, 2003)

Cyanidin-3-glucoside: ^{1}H/^{13}C-NMR chemical shifts (ppm) and coupling constants J (Hz): H-4, 9.03 *s*/135.4; H-6, 6.68 *d* /101.8; H-8, 6.91 *d* /93.6; H-2', 8.07 *d* (2.3)/116.9; H-5', 7.03 *d* (8.7)/115.9; H-6', 8.25 *dd* (8.7,2.3)/126.7;

H-1'', 5.31 *d* (7.7)/102.2; H-2'', 3.64-3.67 *m*/69.5; H-3'', 3.58- 3.60 *m*/77.1; H-4'', 3.56-3.59 *m*/76.6; H-5'' 3.69-3.72 *m*/73.2; H-6'', 3.94 *dd* (12.1,2.2)/60.7.

Cyanidin-3-rutinoside: H-4, 8.95 *s*/137.8; H-6, 6.70 *d* /102.0; H-8, 6.90 *d* /93.7; H-2', 8.07 *d* (2.3)/116.9; H-5', 7.05 *d* (8.7)/116.0; H-6', 8.25 *dd* (8.7,2.3)/126.9; H-1'', 5.23 *d* (7.8)/101.9; H-2''-H-5'', 3.44-3.70 *m*; H-6''a, 4.09 / 66.2, H-6''b, 3.59 / 66.2. Ramnosyl moiety: H-1''', 4.96 d (1.6)/100.6; H-2'''-H-5''' 3.36-3.83 m; H-6'', 1.19 d (6.21)/16.2.

Cyanidin-3-5-diglucoside: H-4, 9.12 *s*/134.5; H-6, 7.07 *bs* /104.3; H-8, 7.07 *bs* /95.9; H-2', 8.05 *d* /117.1; H-5', 7.02 *d* (8.8)/116.1; H-6', 8.32 *dd* (8.8,2.3)/127.6; Glucosyl 1: H-1'', 5.30 *d* (7.9)/102.5; H-2''-H-5'', 3.43-3.67 *m*; H-6''a, 4.00, H-6''b 3.70 /60.8. Glucosyl 2: H-1''', 5.17 *dd* (7.7,3.9) /101.2; 3.39-3.70 *m*; H-6'''a 3.94; H-6'''b 3.78 *m* /61.0.

2.9. Evaluation of thermostability

The thermostability of anthocyanins was evaluated in water bath at different temperatures: 40 °C, 60 °C, and 80 °C. The samples were dissolved in 0.1 M citrate buffer pH 3, and absorbance was measured periodically at 510 nm to analyze the effect of temperature on anthocyanins stability (Sari *et al*., 2015), the first-order reaction rate constants (*k*) and half-time ($t_{1/2}$) values were calculated.

2.10. Evaluation of antioxidant activity

The measurement of antioxidant activity was determined by DPPH assay (Brand-Williams, Cuvelier, & Berset, 1995). In brief, 400 µL of 0.05 mM DPPH was added to 40 µL of the extract, and the decrease in absorbance at 515 nm was recorded every 30 s during 30 min in the dark at room temperature. The antioxidant activity of test compounds was expressed as percentage of DPPH inhibition. The assays were performed on Cary 60 UV-Vis Agilent Technologies spectrophotometer. Solution of 1 % formic acid (v/v) in methanol/water (80:20) was used as a negative control, and cyanidin-3-sinapic-diglucoside-5-glucose as positive control. Test compounds were diluted in 1 % formic acid (v/v) in methanol/water (80:20) to the final concentration of 0.5 mM in accordance to the availability of these anthocyanins after isolation process.

In order to prepare dose-response curves for the IC_{50} determination (concentration of compounds that reduces half the maximum response), a set of standard solutions for each of the compound tested was prepared by serial dilution, and the following concentrations were obtained: 0.900, 0.600, 0.300, 0.150, 0.075, 0.030 and 0.015

mM. For the IC_{50} assay, 40 µL ot the test compound at different concentrations were mixed with 400 µL of 0.05 mM DPPH, and following the 30 min incubation, absorbance was measured at 515 nm.

3. Results and discussion

3.1. Extraction and characterization of cyanidin glycosides

The first part of our studies consisted of the extraction and purification of cyanidin glycosides to perform enzymatic esterification. **Table 1** shows the anthocyanin content and purity following extraction from 5 different plant sources (measured by HPLC at 520 nm). Results indicate that purple corn husk had the highest content of anthocyanins among the test samples. Noteworthy, it was found to be richer in anthocyanins than blueberries when compared to the published data (1.91-4.61 mg/g D.W.)(Castrejón, Eichholz, Rohn, Kroh, & Huyskens-Keil, 2008). Purple corn husk has been known as an important source of anthocyanins, and HPLC analysis showed it contained a mixture of six such compounds(Chung-Ying *et al*., 2008), and that cyanidin-3-(6''-malonyl)glucoside was the predominant structure. Because the main objective of this work was the esterification of cyanidin-3-glucoside (**C3G**), the cyanidin-3-(6''-malonyl)-glucoside was hydrolyzed with 1 % HCl (v/v) for 48 h. As a result, the amount of **C3G** increased from 26.1 % to 64.4 % of total anthocyanin content according to the HPLC analysis. These results also proved that corn-husk, despite having the most complex anthocyanins profile of all samples, showed its great potential as **C3G** source since it could be obtained 16 mg/g of C3G from the hydrolysis of the first extract with 24 mg/g of total anthocyanin content.

Another source of **C3G** was tiliapo husk which total anthocyanin content was assessed to be 2.38 mg/g , a concentration comparable to other fruits (Feng *et al*., 2016). Due to the fact that anthocyanins found in this fruit had not been reported before, the NMR spectra were compared with previous reports (Fernandes *et al*., 2014) to confirm the presence of **C3G** in tiliapo fruit. Since **C3G** extracted from tiliapo husk had higher purity than from corn husk (88 % versus 64 %, respectively), the tiliapo was considered a better source of **C3G** for this study as it provided more controlled reaction medium.

The second test compound, cyanidin-3-rutinoside (**C3R**), was obtained from trueno fruit and plum husk at 1.98 mg/g and 12.46 mg/g, respectively, of total anthocyanin content. Trueno fruit contained only one type of anthocyanin while plum husk had four anthocyanins structures, namely cyanidin-3-glucoside, cyanidin-3-xyloside, cyanidin-3-rutinoside, and peonidin-3-rutinoside (Usenik *et al*., 2009). The HPLC chromatogram obtained for trueno fruit matched with the elution time of **C3R** obtained from the plum husk. Additionally, the

HPLC-MS analysis proved the presence of a molecular ion of *m/z* 595 corresponding to cyanidin-3-rutinoside (Slimestad, Vangdal, & Brede, 2009). **C3R** from plum husk was used for the propose of this study, owing to the higher purity and greater total anthocyanin content in comparison to trueno fruit. However, trueno fruit remains an excellent source of **C3R** in terms of its availability as it is hardly consumed as a fruit.

Finally, cyanidin-3,5-diglucoside (**C35G**) was obtained from bottlebrush flowers, and their anthocyanin content stood at 6.15 mg/g and purity of 67.6 %. Molecular ion of *m/z* 611 and its MS^2 fragmentation to *m/z* 287 in positive ion mode confirmed the presence of **C35G** (Barnes & Schug, 2011); and demonstrated by NMR analysis. Overall, three cyanidin derivatives were obtained to evaluate the effect of glycosyl moiety on enzymatic esterification.

3.2. Evaluation of enzymatic esterification conditions

In this work, lipases were used to catalyze reactions between cyanidin glycosides and different aromatic acyl donors (**Figure 1**). All reactions were carried out at 60 ºC and 300 rpm for 48 h as usually reported in previous works (Cruz *et al*., 2017). The progress of individual reactions was monitored by HPLC, and the formation of new peaks was detected; anthocyanins at 520 nm and all other phenolic compounds at 325 nm. **Figure S1** shows the chromatogram of the reaction between **C3G** and dihydrocinnamic acids (dihydrocinnamic and dihydroferulic acids), in which the retention time of the esters was detected at 25.2 min and 20.4 min, respectively. The conversion of the esterification was calculated from the area of the reaction product.

3.2.1. Effect of solvent

The solubility of the substrates in the reaction media is a fundamental requirement to perform any chemical reaction; therefore, in this investigation it was necessary to make an analysis of solvents that were compatible with each of the substrates participating in the esterification reactions, since anthocyanins are soluble in aqueous solvents while esterification reactions with lipases are favored in organic solvents and with low aqueous activities, so it was also necessary to guarantee the removal of water from the medium through molecular sieves. **Table 2**, last column, shows the conversions obtained for different reaction conditions tested in this study, where the importance of the reaction solvent stands out, with *tert*-butanol with the molecular mesh being the solvent where a higher conversion of anthocyanin ester was achieved, followed by pyridine and 2M2B. This is due to the solubility of the substrates in the reaction medium, mainly of the anthocyanins since in the C3G solubility data described in **Figure S2** it is shown that C3G has a greater zero time solubility with pyridine,

however, when exposed to longer times the C3G begins to degrade. On the contrary with *tert*-butanol the solubility of anthocyanin at zero time is less than 40 % and when the agitation time increases the solubility reaching more than 60 % solubility at 24 hours and even at 72 hours the substrate degradation wasn't detected in this organic solvent; while with 2M2B the solubility overtime does not exceed 40 %. Therefore, to ensure greater efficiency of the transformation of anthocyanin to esterified anthocyanin, it must first be ensured that the products are soluble in the reaction medium and do not degrade.

3.2.2. Effect of the enzyme.

As it was mentioned before, the enzymatic esterification with lipases represented a challenge that needed to be addressed. In the next step, the effect of different lipases and enzyme concentrations were evaluated. Therefore, three commercial enzymes were studied: two *Candida antarctica* lipases and *Candida cylindracea* lipase. According to **Table 2**, seventh column, the *Candida antarctica* from SIGMA-ALDRICH performed better than *Candida cylindracea.*

The results showed in **Table 2,** eighth column, indicate that enzyme concentration of 10 g/L or 20 g/L had no effect on the conversion value. However, it was observed a lower conversion when the enzyme concentration was reduced to 5 g/L. In consequence, a higher enzyme concentration improved the conversion.

3.2.3. Effect of acyl donor

Another parameter studied was the type of acyl donor. The following compounds were included in the study: vinyl ester (vinyl cinnamate **VC**), α-β unsaturated carboxylic acid (cinnamic acid **CA**) and α-β saturated carboxylic acids (dihydrocinnamic acid **DHC,** dihydroferuloyl acid **DHF**, dihydrosinapic acid **DHS**). The results showed in **Table 2**, fifth column**,** indicate that the usage of vinyl ester improved enzymatic conversion by shifting the equilibrium of the reaction towards the product formation because of the acetaldehyde evaporation. On the other hand, the addition of molecular sieves to the reaction media had the same effect when **CA** and **DHC** were used as acyl donors, but the conversion was lower in comparison to the reaction with **VC**. For α-β unsaturated and α-β saturated carboxylic acids as acyl donors, it was found that **DHC** gave higher conversion than **CA**, which is in accordance with the results reported (Cassani, Luna, Navarro, & Castillo, 2007). Additionally, the concentration of acyl donor was evaluated, using an excess of **VC**: 50, 100, and 250 equivalents. The conversion was measured after 48 h, and the best results were obtained when 250 eq. of **VC** was used. For 100 eq of acyl donor, the reaction also occurred although with lower conversion yields, but no progress was

observed when the concentration was below 50 eq. The results obtained are in agreement with previous reports on aromatic esterification, where a large excess of acyl donor (Enaud, Humeau, Piffaut, & Girardin, 2004) or modest excess(Stevenson, Wibisono, Jensen, Stanley, & Cooney, 2006) was used to perform the reaction. To sum up, enzymatic esterification of cyanidin-3-glucoside required a huge excess of acyl donor, which was the critical factor in the lipase catalyzed reaction.

3.2.4. Effect of anthocyanin glycoside.

Once the critical factors of the enzymatic esterification of cyanidin-3-glucoside were established, it was decided to expand the study towards other cyanidin glycosides, such as cyanidin-3,5-diglucoside that contains two glucoses, one in position 3 and the other in position 5; and cyanidin-3-rutinoside that contains disaccharide in the position 3 (6-O-α-L-rhamnosyl-D-glucose). The results obtained (**+**) indicated that the esterification of cyanidin-3,5-diglucoside had the highest conversion, followed by cyanidin-3-glucoside. The lowest conversion was obtained for cyanidin-3-rutinoside as it lacks the primary alcohol group and therefore, it exhibits less reactivity. Oppositely, the success on the esterification of cyanidin-3,5-diglucoside could be explained by the presence of two primary alcohol groups, although the esterification at both glucosides was not detected.

Finally, **Table 3** summarized the HPLC results obtained for the extracted cyanidin-3-glucoside and its esters together with the results of MS analysis. The molecular ion $[M]^+$ of the chromatographic peak products corresponds to the respective cyanidin-3-(6''-dihydrocinnamoyl)glucoside, cyanidin-3-(6''-cinnamoyl)glucoside, cyanidin-3-(4'''-cinnamoyl)rutinoside and cyanidin-3-(6''-cinnamoyl)glucoside,5-glucoside and MS^2 fragments indicate that esterification occurred only on the glycoside moiety.

3.3. Structural elucidation by NMR.

Five acylated anthocyanin products were isolated by silica gel column using a mixture of ethyl acetate : methanol : formic acid (80:10:10) as an eluent. Chemical structure of the novel products was determined by means of 1D and 2D NMR techniques (**Table S1**) (Cruz *et al*., 2018), (Pérignon, Lecomte, Pina, Renault, Simonneau-Deve, & Villeneuve, 2013),(Moloney, Robbins, Collins, Kondo, Yoshida, & Dangles, 2018). The acylation took place at the position 6''-OH of glucose moieties, according to the observed downfield shifts of the corresponding chemical shifts: 3.9 to 4.5 ppm in the ^1H spectrum, and from 60 to 63 ppm in the ^{13}C spectrum and also confirmed by the HMBC spectrum. The NMR spectra of cyanidin-3,5-diglucoside cinnamate ester showed that acylation occurred

preferentially on the primary alcohol of the glucose in the position 3, according to the observed downfield shift from 3.70 to 4.5ppm.

These resonance spectra also show us the interactions of stacking π-π between the phenolic nuclei (Trouillas, 2016) in the case of compounds C5 and C8 the chemical displacements of hydrogen corresponding to H6, H4 and H8 of the aglycone move to a low field compared to native anthocyanin (C3G) while in compounds C4, C7 and C9 the chemical shifts of hydrogen corresponding to H6, H4 and H8 of the aglycone move to a high field compared to native anthocyanin (C3G) . It should be noted that this effect is more noticeable in C9 (anthocyanin acylated with DHS), so it can be suggested that in this compound the interactions between both phenolic nuclei are closer than in the rest of the esterified anthocyanins.

3.4. Thermostability evaluation.

Thermostability of C3G and its corresponding purified esters was evaluated at 40, 60, and 85 °C. As shown in **Table 4**, the kinetic rate constant (k) and half-life parameter indicated that cinnamoyl, dihydroferuloyl and dihydrosinapoyl cyanidin esters (**4, 8** and **9)** demonstrated improved stability than **C3G,** with the most pronounced effect observed at 85 °C. However, in case of dihydrocinnamoyl cyanidin ester (**7**), the kinetic rate constant (k) was higher than **C3G** but showed shorter half-life value in comparison to **C3G.** The obtained data suggest that both the double bond presented in the cinnamic acids and the substituents in the ring are responsible for copigmentation and therefore the thermostability. Till present, no studies on the evaluation of the thermostability of the anthocyanin esters with dihydrocinnamic acids have been reported, however, previous reports demonstrated that the esterification improves the thermostability (Yang, Kortesniemi, Ma, Zheng, & Yang, 2019)(Sari *et al*., 2015). To sum up, compound **9** that corresponds to dihydrosinapoyl cyanidin ester has improved considerably the thermostability of C3G, becoming an option to stabilize this most abundant anthocyanin.

3.5. Antioxidant activity

The radical scavenging activity of the purified cyanidin-3-glucoside acylated esters was evaluated. **Figure S3** shows that compound **9** and dihydrosinapic acid had the highest inhibition potential, followed by the red cabbage anthocyanin which exhibited similar percentage of DPPH inhibition to that presented by the compound **4**. Compound **8** showed 30 % less antioxidant activity than compound **1** and dihydroferulic acid. In **Table 5,** the

IC_{50} values of the test compounds were compiled, and the results obtained suggest that the antioxidant activity depends on the acyl donor linked to the cyanidin-3-glucoside.

Furthermore, compound **9** had the highest radical scavenging activity, exceeding even red cabbage anthocyanin which possess a sinapic acid moiety and more than one of glycosyl moieties. On the other hand, the esterification of the cyanidin-3-glucoside with the dihydrocinnamic acid (compound **7**) reduced the antioxidant activity of the cyanidin-3-glucoside. This effect could be explained by the rotation of the dihydrocinnamic moiety that may interfere with access to DPPH due to steric hindrance (Lue, Skall, Jacobsen, Hellgren, Guo, & Xu, 2010). These results showed that instauration in the acyl moiety has an important role in that antioxidant activity, and the esterification improved the antioxidant activity in comparison with the cyanidin-3-glucoside.

4. Conclusions

In summary, cyanidin-3-glucoside, cyanidin-3-rutinoside, and cyanidin-3,5-diglucoside were successfully extracted from underutilized plant sources, purified and identified by HPLC-MS. Furthermore, cyanidin-3-glucoside was characterized in tiliapo (*Sideroxylon palmeri*) and cyanidin-3-rutinoside in trueno (*Ligustrum japonicum*) fruits. All cyanidin glycosides were enzymatically esterified after establishing the best reaction conditions. Optimal reaction conditions involved *tert*-butanol as reaction media, 20 g/L of *Candida Antarctica*, and 250 equivalents of vinyl cinnamate as acyl donor which was found to be a critical parameter. Under those conditions, five new acylated anthocyanins were synthesized, purified and characterized: cyanidin-3-(4'''-cinnamoyl)rutinoside (**6**), cyanidin-3,5-(6''-cinnamoyl)diglucoside (**5**), cyanidin -3-(6''-dihydrocinnamoyl)glucoside (**7**), cyanidin-3-(6''-dihydroferuloyl)glucoside (**8**), and cyanidin-3-(6''-dihydrosinapoyl)glucoside (**9**). Overall, it was also demonstrated that cyanidin-3-(6''-dihydroferuloyl)glucoside (**8**) and cyanidin-3-(6''-dihydrosinapoyl)glucoside (**9**) obtained by enzymatic esterification exhibited better thermostability than cyanidin-3-glucoside, and its antioxidant activity was improved with dihydrosinapoyl and cinnamoyl residues. This work provided a systematic study for the preparation of a series of acylated anthocyanins from underutilized sources. In further work, the stability of these compounds at different pH will be determined.

Notes

The authors declare no competing financial interest

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509 **FIGURE CAPTIONS**

510 **Figure 1.** Esterification reactions of cyanidin glycosides

511 **Table 1. Total anthocyanin content and purity of cyanidin glycosides from different plant sources.**

Source	Scientific name	Total anthocyanins (mg/g D.W.)	Anthocyanin	Cyanidin obtained (mg)	Purity (%)
Purple corn-husk	*Zea mays*	24.87±1.03	Cyanidin-3-glucoside	120	64.4
Tiliapo	*Syderoxylon palmeri*	2.38±0.05	Cyanidin-3-glucoside	60	88.6
Trueno	*Ligustrum japonicum*	1.98±0.57	Cyanidin-3-rutinoside	20	40.0
Plum husk	*Prunus domestica*	12.46±1.58	Cyanidin-3-rutinoside	16	83.1
Bottlebrush flower	*Callistemon citrinus*	6.15±0.39	Cyanidin-3,5-diglucoside	13	67.6

512

513 **Table 2. Reaction conditions of enzymatic esterification of cyanidin glycosides.**

Reaction	Solvent	Cyanidin	Cyanidin (mg)	Acyl donor	Concentration AD (eq.)	Lipase	Concentration (g(/L)	Molecular sieves 4A	Conversion (%)
1	Pyridine	C3G	5	VC	250	CAS	20	100	70.3
2	Pyridine	C3G	5	VC	250	CAS	20	0	54.2
3	2M2B	C3G	5	VC	250	CAS	20	100	59.5
4	2M2B	C3G	5	VC	250	CAS	20	0	36.4
5	*Tert*-butanol	C3G	5	VC	250	CAS	20	100	74.1
6	*Tert*-butanol	C3G	5	VC	250	CAS	20	0	38.1
7	*Tert*-butanol	C3G	5	VC	250	CAS	10	100	59.9
8	*Tert*-butanol	C3G	5	VC	250	CAS	5	100	21.3
9	*Tert*-butanol	C3G	5	VC	250	CLECTA	20	100	59.9
10	*Tert*-butanol	C3G	5	VC	250	CLECTA	10	100	49.1
11	*Tert*-butanol	C3G	5	VC	250	CLECTA	5	100	5.3
12	*Tert*-butanol	C3G	5	VC	250	CYL	20	100	21.3
13	*Tert*-butanol	C3G	5	VC	250	CYL	10	100	9.4
14	*Tert*-butanol	C3G	5	VC	250	CYL	5	100	1.8
15	*Tert*-butanol	C3G	5	VC	100	CAS	20	100	41.7
16	*Tert*-butanol	C3G	5	VC	50	CAS	20	100	24.4
17	*Tert*-butanol	C3G	5	DHC	250	CAS	20	100	67.3
18	*Tert*-butanol	C3G	5	DHC	100	CAS	20	100	20.6
19	*Tert*-butanol	C3G	5	DHC	50	CAS	20	100	1.1
20	*Tert*-butanol	C3G	5	DHF	250	CAS	20	100	45.0
21	*Tert*-butanol	C3G	5	DHS	250	CAS	20	100	21.8
22	*Tert*-butanol	C3G	5	CA	250	CAS	20	100	18.1
23	*Tert*-butanol	C3G	5	CA	100	CAS	20	100	0
24	*Tert*-butanol	C3G	5	CA	50	CAS	20	100	0
25	*Tert*-butanol	C3R	5	VC	250	CAS	20	100	45.5
26	*Tert*-butanol	C35D	5	VC	250	CAS	20	100	85.7

C3G**, cyanidin-3-glucoside; **C3R**, cyanidin-3-rutinoside; **C35D**, cyanidin-3,5-diglucoside; **VC**, vinyl cinnamate; **CA**, cinnamic acid; **DHC**, dihydrocinnamic acid; **DHF dihydroferuloyl acid, DHS dihydrosinapoyl acid, CAS, lipase of Candida antartica from SIGMA-ALDRICH, CLECTA lipase of Candida antartica from C-lecta industry, CYL, lipase of Candida cylindracea
Reaction condition: 300 rpm, 60°C, 48 h.

514

515 **Table 3. HPLC-mass spectrometry and percentage of conversion.**

Compound	Retention time (min)	Molecular Ion $[M]^+$ (m/z)	MS^2
(**1**) Cyanidin-3-glucoside	11.6	449	287
(**2**) Cyanidin-3-rutinoside	13.6	595	287
(**3**) Cyanidin-3,5-diglucoside	9.9	611	449, 287
(**4**) Cyanidin-3-(6''-cinnamoyl)glucoside	24.8	579	287
(**5**) Cyanidin-3-(6''-cinnamoyl)glucoside-5-glucoside	23.8	741	449, 287
(**6**) Cyanidin-3-(4''-cinnamoyl)rutinoside	24.1	725	287
(**7**) Cyanidin-3-(6''-dihydrocinnamoyl)glucoside	25.6	581	287
(**8**) Cyanidin-3-(6''-dihydroferuloyl)glucoside	20.4	627	287
(**9**) Cyanidin-3-(6''-dihydrosinapoyl)glucoside	16.6	657	287

516

517 **Table 4. Kinetic parameters for the thermal degradation of acylated anthocyanins obtained by enzymatic**

518 **esterification**

Compound	Temperature (°C)	*k* (h)	$t_{1/2}$(s)
1	40	0.0285 (0.9544)	24.3
	60	0.0657 (0.9820)	10.5
	85	0.1909 (0.9985)	3.6
4	40	0.0121 (0.9540)	57.0
	60	0.0133 (0.9773)	52.0
	85	0.0563 (0.9365)	12.3
7	40	0.0468 (0.9985)	14.8
	60	0.1751 (0.9883)	4.0
	85	0.4947 (0.9665)	1.4
8	40	0.0228 (0.9087)	30.4
	60	0.0512 (0.8876)	13.5
	85	0.1391 (0.9678)	5.0
9	40	0.0321 (0.9143)	22.2
	60	0.0508 (0.9639)	13.6
	85	0.0728 (0.9939)	9.5
Red cabbage anthocyanin	40	0.0224 (0.8148)	30.9
	60	0.0712 (0.8070)	9.7
	85	0.1657 (0.9562)	4.2

Numbers in parentheses are the correlation coefficients

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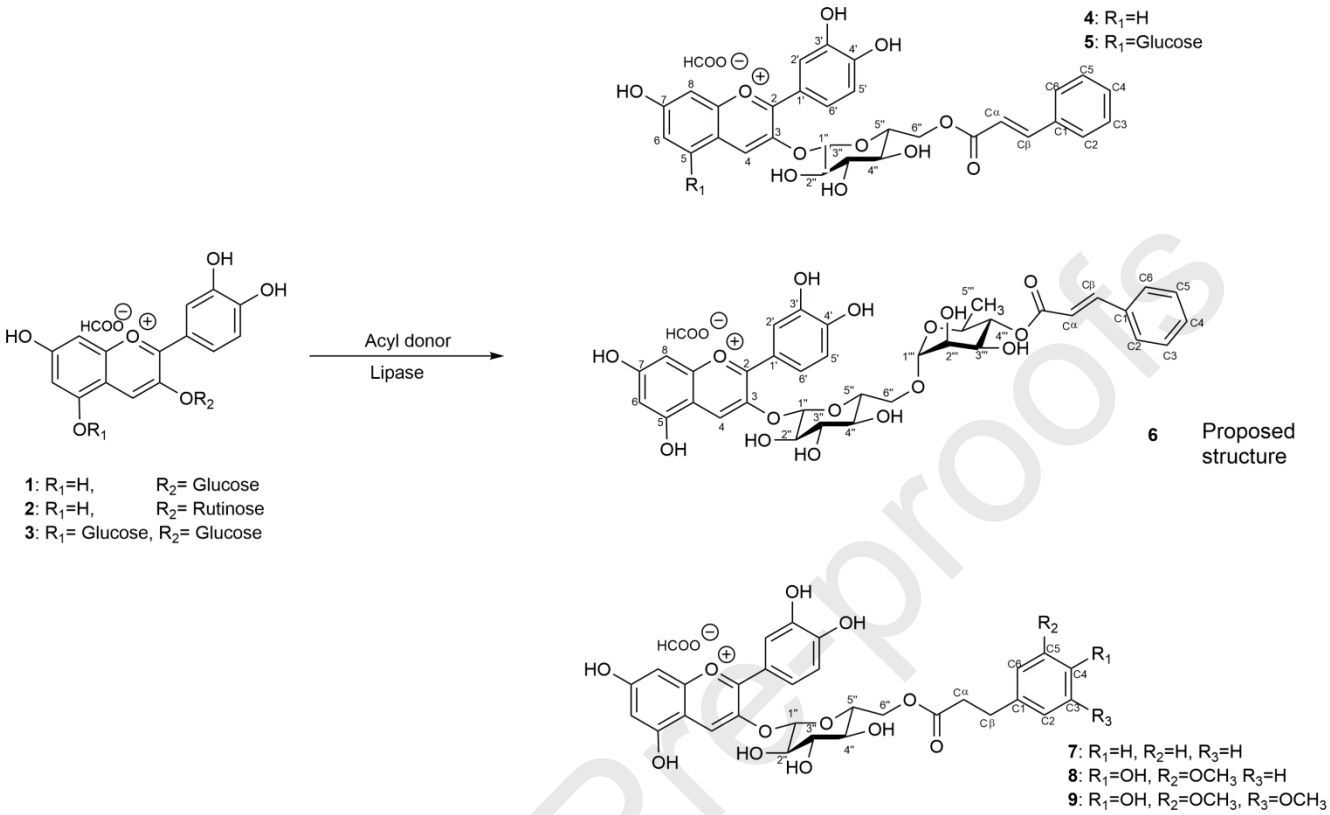
520

Table 5. Comparison of the antioxidant activity (IC_{50}) of the anthocyanins and acyl donors.

Compound	IC_{50} (mM)
1	0.265 ±0.019
4	0.119 ±0.008
7	N/A
8	0.909 ± 0.127
9	0.027 ± 0.003
Dihydroferulic acid	0.564 ±0.073
Dihydrosinapic acid	0.151 ± 0.013
Dihydrocinnamic acid	N/A
Cinnamic acid	N/A
Red cabbage anthocyanin	0.036 ± 0.004

N/A: The compound does not show antioxidant activity

Figure 1.



- Evaluation of five underutilized plants to obtain sources of cyanidin glycosides
- Reaction conditions of enzymatic esterification to anthocyanins for the food industry
- Synthesis of four new anthocyanins derived from dihydrocinnamic acids
- Development of cyanidin esters with more thermostability and antioxidant activity

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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