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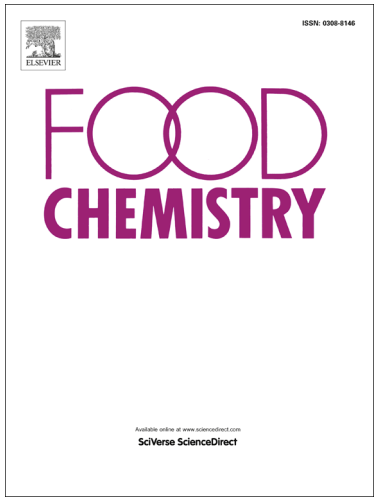

Short communication

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Selective enzymatic lipophilization of anthocyanin glucosides from blackcurrant (*Ribes nigrum* L.) skin extract and characterization of esterified anthocyanins

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Abstract

Anthocyanins (ANC) are hydrophilic and water-soluble polyphenolic plant pigments. The current barriers to successful application of ANC in food, cosmetic and pharmaceutical industries are predominantly related to performance, stability, formulation properties, and color. Enzymatic acylation of ANC could increase their stability without compromising bioactivity and chromatic features. Lipophilization of ANC-rich blackcurrant skin extract with *Candida antarctica* lipase B and octanoic acid was selective to cyanidin and delphinidin glucosides, but not the corresponding rutinosides. The reaction was chemo- and regioselective for acylation at the primary alcohol of the glucose moieties, greatly facilitating separation of the different glycoside derivatives.

Keywords: blackcurrant extract; fatty acids; enzymatic catalysis; selective lipophilization; mass spectrometry; anthocyanin; glycosylation; renewable effect chemicals; *Ribes nigrum*.

1. Introduction

Anthocyanins (ANC) are the largest group of polyphenolic pigments in the plant kingdom, and are hydrophilic and water-soluble, primarily as a result of their glycosylation. Their widely-reported biological properties (Fernandes *et al*., 2010; Fernandes *et al*., 2013; Évora *et al*., 2017) and inherent color has resulted in significant research in recent years on the application of ANC and their derivatives in the food, cosmetic and pharmaceutical industries (Rose *et al*., 2018). The current barriers to successful application of ANC in these fields are predominantly related to performance, stability, formulation properties, and color. Some plants make acylated ANC derivatives, which changes certain properties; glycosides of ANC in black carrots (*Daucus carota* subsp. *sativus* var. *atrorubens* Alef.) are acylated with hydroxycinnamic and hydroxybenzoic acids (Coultate & Blackburn, 2018), increasing the range of food products in which these colorants can be used; red cabbage (*Brassica oleracea* L. var. *capitata* f. *rubra*) extracts also contain highly acylated ANC (Ahmadiani *et al*., 2014). These acylated

ANC have increased stability as a result of intramolecular copigmentation effects, which affords greater heat stability at higher pH values, in comparison with non-acylated anthocyanins (Coultate & Blackburn, 2018). Acylated ANC also have decreased water solubility, due to the greatly reduced hydrophilicity of an ester compared with an alcohol.

However, all ANCs are sparingly soluble in more lipophilic media, which compromises their effective application in lipophilic systems, such as fats, oils, lipid-based foods or cosmetic formulas and emulsions. A solution to increase the hydrophobic nature of flavonoids, to render them suitable for such applications, involves the esterification of their hydroxyl groups with fatty acids and other moieties (Kontogianni *et al*., 2003; Chebil *et al*., 2007; Viskupicova *et al*., 2010; Zhu *et al*., 2014). Studies have shown the chemical esterification of quercetin-3-*O*-glucoside (Q3G) to be non-chemo- and non-regioselective, leading to functionalization of phenolic groups, and decreasing antioxidant activity. However, enzymatic acylation of Q3G was shown to be more chemo- and regioselective and to enhance not only flavonoids' solubility in various non-aqueous media, but also their stability and antioxidant activity (Salem *et al*., 2010; Bhullar *et al*., 2014). Successful enzymatic acylation of the glucose primary –OH of Q3G using *Candida antarctica* lipase B (*Ca*lB) has been reported; lipase-catalyzed regioselective acylation of Q3G with cinnamic acid and *p*-coumaric acid significantly increased thermal stability and light fastness, respectively, compared to Q3G (Ishihara & Nakajima, 2003). Recently, Vaisali *et al*. (2017) demonstrated immobilized *Candida antarctica* lipase mediated rutin fatty ester synthesis in both *tert*-butanol and acetone; they subsequently demonstrated that the rutin ester showed greater reduction in primary and secondary oxidation of refined sardine oil compared to the more hydrophilic rutin, after storage for 20 days (Vaisali *et al*., 2018).

When compared to other flavonoids, chemical modification of ANCs is highly challenging because of the various dynamic equilibrium forms in solution (Brouillard & Delaporte, 1977), their low solubility in organic solvents, and their susceptibility to degradation in neutral and basic conditions,

due to the hydration of the reactive cationic flavylium ring, or its derivatives. Furthermore, the production of significant quantities of purified ANC is difficult and expensive.

Previous work has reported the chemical acylation of a pure malvidin-3-*O*-glucoside (Mv3glc) to yield the stearic acid derivative, using stearoyl chloride in anhydrous acetonitrile (Cruz *et al*., 2015); however, the reaction was not regioselective and a complex mixture of mono-, di-, and tri-ester derivatives was obtained. In contrast, the regioselective synthesis of a pure malvidin-3-glucoside-oleic acid ester derivative was achieved by enzymatic catalysis with *Ca*lB, yielding only one ester product (21% conversion) (Cruz *et al*., 2016). The more lipophilic derivative did not show any reduction in antioxidant potential and protected more effectively a lipidic substrate from oxidation. Enzymatic catalysis using *Ca*lB in reactions between pure Mv3glc and different fatty acids (C_4, C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16}) gives yields of acylated ANC of 22–40%, which could be separated by column chromatography (Cruz *et al*., 2017). All acylated ANCs with fatty acid chain length C8 and above showed particularly enhanced lipophilicity and preferentially partitioned into octanol in an octanol-water biphasic system. A small increase in λ_{max} by up to 12 nm was observed, increasing with increasing chain length.

Taking into account the specificity of *Ca*lB to catalyze the formation of single monoesters of anthocyanin-3-*O*-glucosides at the primary C6''–OH, this enzymatic reaction could be attempted on ANC-rich extracts, which may also allow separation based on carefully tuned physical properties. In the work described herein, the aim was to perform lipophilization of a complex blackcurrant extract to provide acylated ANC from a mixture of polyphenols, and to explore this technique as a new tool to selectively separate ANC glucoside derivatives from the structurally related rutinosides. Enzymatic acylation of a complex mixture of ANC and other polyphenols, which would be expected from a commercial extraction process, has not been previously reported.

2. Materials and methods

2.1. Materials

Blackcurrant pomace was obtained from GlaxoSmithKline, Brentford, UK and more recently from A&R House Ltd., Bleadon, UK. The raw fruit grown in the UK had been pressed in production of blackcurrant cordial (*Ribena*) (Lucozade Ribena Suntory Ltd, 2018). The crude waste is referred to as pomace, which comprises the fruit skins (*ca*. 50% *w/w*), seeds (*ca*. 45% *w/w*) and extraneous matter (e.g., berry stalks, *ca*. 5% *w/w*). Seeds are separated from this pomace and unwanted stalks removed; the subsequent material received was predominantly dried blackcurrant fruit skins, used without any further modification. Amberlite XAD7HP was obtained from Rohm & Haas (Staines, UK). Delphinidin-3-*O*-glucoside was purchased from Polyphenols AS, Sandnes, Norway. Reversed-phase C18 (RP-C18) silica gel (40–63 μm) LiChroprep was provided by Merck (Darmstadt, Germany). Fatty acids, lipase acrylic resin from *Candida antarctica* lipase B (≥ 5000 U/g, recombinant, expressed in *Aspergillus niger*), molecular sieves 4Å, 2-methyl-2-butanol, and other general purpose chemicals were obtained from Sigma-Aldrich (Madrid, Spain).

2.2. Extraction of blackcurrant

Blackcurrant (*Ribes nigrum* L.) fruit skins (60 g) were immersed in water (1200 mL) acidified 0.01% *v/v* with conc. HCl and stirred very gently by magnetic follower at room temperature for 2 hours. The resulting aqueous extract was subjected to solid-phase extraction (SPE) using a column of Amberlite XAD-7HP (120 g) using acidified water (0.01% *v/v* conc. HCl), until a colorless eluent was obtained, and then acidified ethanol (0.01% *v/v* conc. HCl). The collected ethanol fractions were combined and concentrated under vacuum on a rotary evaporator, and then subjected to high vacuum to remove trace solvent, yielding a dark violet amorphous solid (880 mg, yield 2.2%), which could be powdered by grinding. In recent research (Farooque *et al*., 2018), this post-SPE blackcurrant extract was characterized by HPLC, mass spectrometry, IR, NMR and UV-Vis spectroscopy. It was determined

that the extract comprised monomeric ANC (54.7%), polymeric ANC (18%), flavonols (17.1%), and hydrocinnamic acid derivatives (9.5%).

2.3. Purification of blackcurrant extract

Crude blackcurrant extract (500 mg) was dissolved in 100 mL of acidified water with 2% HCl and extracted with ethyl acetate (3 × 100 mL). The aqueous fraction was concentrated and purified in a Buchner funnel loaded with RP-C18 silica gel. A fraction of water/methanol 70:30 (*v*/*v*) acidified with 2% HCl was used to elute the ANC. After MeOH evaporation, the aqueous fraction was freeze-dried, yielding a dark red solid (218 mg).

2.4. Synthesis and isolation of ANC-*fatty acid derivatives*

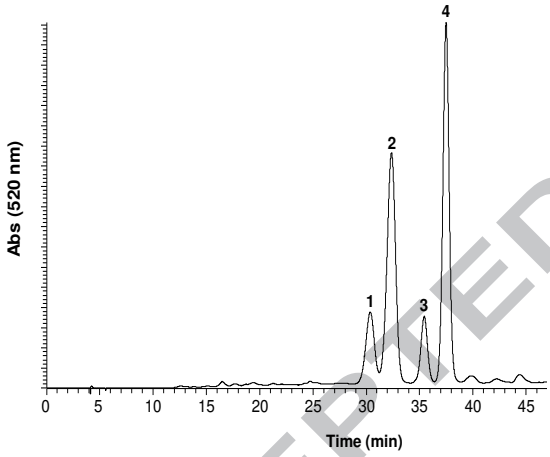
To a flask was successively added 60 mg of purified blackcurrant ANC-rich extract, activated 4Å molecular sieves (100 g L^{-1}) and dry acetonitrile:DMSO 10:1 (*v*/*v*) (5 mL). To this solution, octanoic acid was added (100 eq.). All controls were performed and the control reaction without adding enzyme did not yield the esterified products. The reactions started with the addition of the *Ca*lB enzyme (20 g L^{-1}). The reactions were stirred at 60 ºC over a 9 h period and their progression was followed by HPLC-DAD, as demonstrated in earlier research (Cruz *et al*., 2017). At the maximum products formation, the reaction was filtered to remove the molecular sieves, *Ca*lB and the solvent was evaporated under vacuum. The residue was re-dissolved in acidified methanol (with 2% HCl solution) and the excess of fatty acid and other impurities were extracted with heptane and hexane. Methanol fraction was concentrated, diluted in 20% of aqueous acidified MeOH and purified by chromatography on a column loaded with RP-C18 silica gel (150 mm × 16 mm i.d.). The starting material was recovered with 30% aqueous MeOH and the lipophilic products were isolated with 70% aqueous acidified MeOH. MeOH was removed by evaporation and the pigments were lyophilized and stored at

–18 ºC until use. Reactions performed with 60 mg of purified blackcurrant ANC-rich extract starting material yielded sufficient product to carry out all analyses.

3. Results and discussion

3.1. Characterization of blackcurrant extract

The ANC components of this post-SPE blackcurrant extract were characterized by LC-DAD/ESI-MS (Figure 1). The extract was composed of four monomeric ANC: delphinidin-3-*O*-glucoside (Dp3glc; **1**; 15.7%); delphinidin-3-*O*-rutinoside (Dp3rut; **2**; 43.3%); cyanidin-3-*O*-glucoside (Cy3glc; **3**; 7.0%); and cyanidin-3-*O*-rutinoside (Cy3rut; **4**; 34.0%).



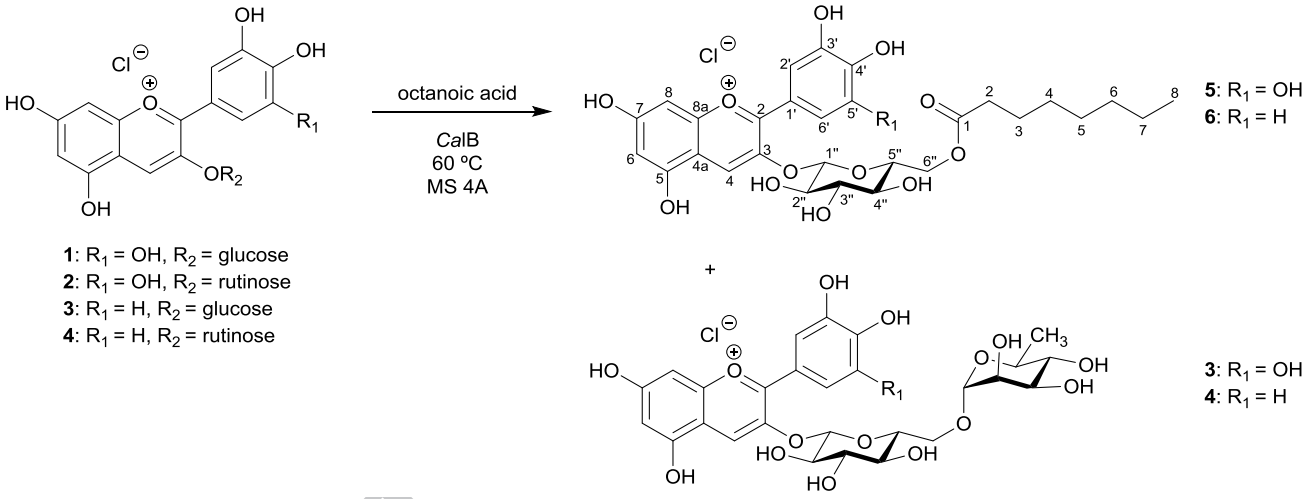
ANC	RT (min)	$[M]^+$ *m/z*	MS^2 *m/z*	Relative %[a]
Dp3glc (**1**)	30.38	465	303 (-162)	15.7
Dp3rut (**2**)	32.37	611	303 (-308)	43.3
Cy3glc (**3**)	35.43	449	287 (-162)	7.0
Cy3rut (**4**)	37.49	595	287 (-308)	34.0

Figure 1. Analysis of blackcurrant extract by LC-DAD/ESI-MS using a RP-C18 column in positive ion mode and determination of relative percentages of ANC. [a]determined by peak area.

3.2. Enzymatic esterification

Reactions between the post-SPE blackcurrant ANC-rich extract and octanoic acid were attempted using enzymatic catalysis, but assays indicated that the acylated ANC derivatives were not formed. This is possibly due to the complexity of the extract containing other compounds, including flavonols (glycosylated and non-glycosylated) and hydroxycinnamic acids, which may compete with or interfere

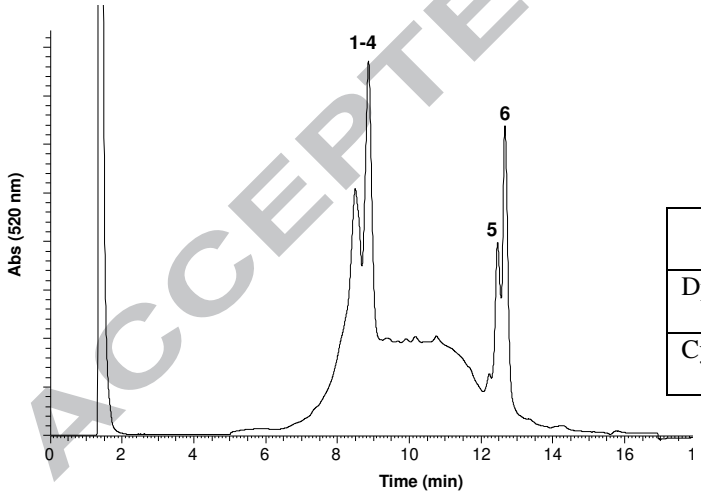
with the biocatalyst's activity. Accordingly, further purification procedures were performed, in order to obtain an extract containing only the four ANCs (Farooque *et al*., 2018): ethyl acetate extraction of an aqueous acidic solution of the original extract removed flavonols and hydroxycinnamic acids, and adsorption onto RP-C18 silica gel and further elution with water allowed removal of any remaining sugars and inorganic salts. The ANCs were then eluted with acidified ethanol and concentrated *in vacuo*. This purified ANC-only extract was subsequently used in esterification reactions with octanoic acid catalyzed by *Ca*lB (Scheme 1).



Scheme 1. Enzymatic esterification reactions of the ANC-rich extract from blackcurrant with octanoic acid by *Ca*lB. Reaction shows exclusive acylation of the glucoside moiety; the rutinoside moiety is not acylated.

The solubility of the highly hydrophilic ANCs in anhydrous solvents is particularly low and hence challenging for acylation reactions. Pure solvents, including acetone, acetonitrile and 2-methyl-2-butanol, and binary mixtures, namely acetone:2-methyl-2-butanol (2:1 *v/v*), acetone:DMSO (10:1 *v/v*), and 2-methyl-2-butanol:DMSO (10:1 *v/v*) were investigated. It is widely described that polar aprotic solvents such as DMF, DMSO and THF are not a good choice for these kinds of biocatalytic reactions

because of the tendency to inactivate the enzymes (de Araújo *et al*., 2017); however, in some cases using a co-solvent at low percentages (up to 20%) can enhance these enzymatic reactions (Ferrer *et al*., 1999). The best results were achieved using acetonitrile:DMSO (10:1 *v/v*). The progress of the reaction was followed by HPLC-DAD and the formation of new chromatographic peaks was detected (Figure 2); reaching maximum conversion after 9 h. Chromatograms from aliquots taken at earlier time intervals in the esterification reaction were qualitatively similar, but with lower intensity peaks in terms of the C8 esterified products. The use of molecular sieves to capture water liberated during the transformation was essential to displace the equilibrium in favor of the newly formed ester. The optimal temperature for the *Cal*B esterification in these conditions was found to be 60 °C. The reaction mixture was quenched with acidified methanol. The mixture was then filtered to remove the solid-supported enzyme and molecular sieves from liquor mixture. A liquid/liquid partitioning work-up with hexane allowed the removal of excess fatty acid, and the methanol layer was then analyzed by LC-DAD/ESI-MS in positive ion mode.



ANC	RT (min)	$[M]^+$ *m/z*	MS^2 *m/z*
Dp3glc-C8 (**5**)	12.46	591	303 (-288)
Cy3glc-C8 (**6**)	12.67	575	287 (-288)

Figure 2. Analysis of lipophilization reaction of blackcurrant extract by LC-DAD/ESI-MS using a RP-C18 column in positive ion mode.

The molecular ion $[M]^+$ of products correspond only to the anthocyanin-3-glucoside-octanoate conjugates, namely delphinidin-3-glucoside-6''-*O*-octanoate (Dp3glc-C8; **5**) and cyanidin-3-glucoside-6''-*O*-octanoate (Cy3glc-C8; **6**). The MS^2 fragment indicated that the mono-esterification occurred on the glucose residue, and no acylated rutinoside products were detected due to the absence of what is presumably the more reactive primary alcohol on the disaccharide unit of the rutinoside. The acylation reactions occur regioselectively on the most reactive hydroxyl group of the sugar (the primary alcohol), as was previously demonstrated by formation of an Mv3glc-oleic acid conjugate (Cruz *et al*., 2015). It was subsequently possible to separate the unreacted ANC (30% aqueous methanol) from the lipophilized products (70% acidified aqueous methanol) using C18 gel column chromatography, giving yields of around 12% (Figure 3).

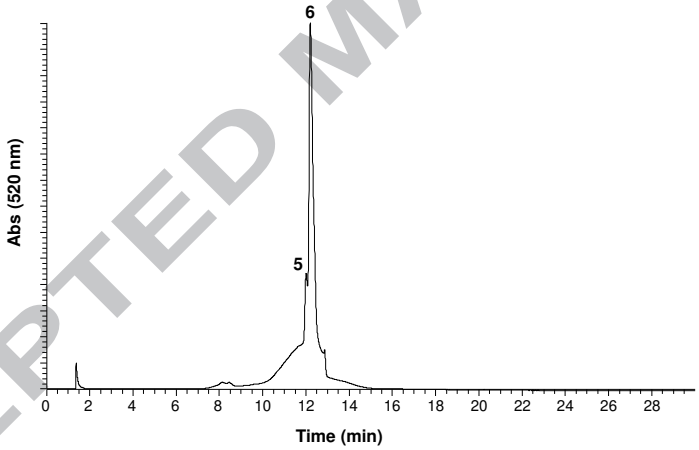


Figure 3. Analysis of the fraction of 70% of aqueous methanol by LC-DAD/ESI-MS using a RP-C18 column in positive ion mode.

The products were further characterized by NMR using 1D and 2D techniques (COSY, HSQC and HMBC) in DMSO-d_6/TFA 9:1 (See supporting information for chromatograms). NMR spectra of the post-SPE blackcurrant extract and the further purified extract before reaction can be seen in Farooque *et al*. (2018). As shown in Table 1, it was possible to assign all protons and carbons to the

two acylated glucosides. ^{13}C NMR spectra confirmed that acylation took place at the primary hydroxyl group of glucose because of the C6'' signal downfield to 63.5 ppm, as already reported for analogous compounds (Cruz *et al*., 2016). Using NMR, it was determined that the ratio between Cy3glc-C8 and Dp3glc-C8 is 3:1, which is interesting considering the relative ratio of Cy3glc and Dp3glc in the extract, is approximately 0.5:1. This suggests that Cy3glc is more preferentially esterified over Dp3glc, which suggests the additional –OH group on delphinidin may exert some steric or electronic effect that decreases the efficiency of the esterification of the glucoside moiety.

Table 1. ^{1}H and ^{13}C NMR data of Cy3glc-C8 and Dp3glc-C8 conjugates in DMSO-d_6/TFA (9:1).[a]

Position	**Cy3glc-C8 (5)**		**Dp3glc-C8 (6)**	
	δ (^{1}H); *J* (Hz)	**δ (^{13}C)**	**δ (^{1}H); *J* (Hz)**	**δ (^{13}C)**
2C	-	161.7	-	161.4
3C	-	144.0	-	144.0
4C	8.79; s	134.5	8.73; s	133.5
4aA	-	111.6	-	111.6
5A	-	157.7	-	157.7
6A	6.70; d, 1.9	102.3	6.69; d, 1.9	102.7
7A	-	168.9	-	168.9
8A	6.88; d, 1.9	94.3	6.83; d, 1.9	94.1
8aA	-	156.0	-	156.0
1'B	-	119.5	-	119.5
2'B	7.97; d, 2.3	117.5	7.70; s	111.5
6'B	8.21; dd, 2.3/8.5	126.9	7.70; s	111.5
3'B	-	146.2	-	143.7
4'B	-	154.6	-	146.5
5'B	7.00; d, 8.5	116.8	-	143.7
Glucose				

1''	5.40; d, 7.7	101.6	5.43; d, 7.7	101.6
2''	3.52; m	73.1	3.59; m	72.9
3''	3.43; m	76.4	3.39; m	76.4
4''	3.23; m	70.2	3.20; m	70.2
5''	3.77; m	74.5	3.81; m	74.5
6''a	4.35; m	63.5	4.30; m	63.5
6''b	4.05; m	63.5	3.97; m	63.5
Caprylic acid				
1 C=O	-	173.1	-	174.8
2 CH_2	2.21; *	33.5	2.14; *	33.5
3 CH_2	1.49, *	24.6	1.35, *	24.6
4-7 $(CH_2)_4$	1.21, *	22.1	1.11, *	22.1
8 CH_3	0.83; t, 7.1	13.8	0.76; t, 7.1	13.8

[a]Key: s, singlet; m, multiplet; d, doublet; t, triplet; dd, double of doublets; *unresolved.

4. Conclusions

In this work, the enzymatic lipophilization of a mixture of ANC extracted from waste blackcurrant (*Ribes nigrum* L.) fruit skins, composed of rutinosides and glucosides of cyanidin and delphinidin, with octanoic acid was performed. The results obtained showed a selective and preferential enzymatic acylation of cyanidin and delphinidin glucosides, whereas the rutinosides were not acylated, most probably due to the absence of a primary hydroxyl group in the saccharide moiety. This lipophilization method could be used and explored as a new technique to separate ANCs with different glycosylation patterns in mixtures otherwise difficult to separate.

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Highlights

- Complex mixtures of anthocyanins (ANCs) are successfully esterified with enzymes.
- Esterification with lipase B is chemo- and regioselective at primary glucose –OH.
- Cyanidin and delphinidin glucosides selectively esterified; rutinosides are not.
- Lipophilization of anthocyanin-rich extract with octanoic acid is successful.
- Lipophilization a new technique to separate ANCs with different glycosylation.