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Enzymatic Hemisynthesis of Metabolites and Conjugates of Anthocyanins

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This work aims to study the phase II metabolization of anthocyanins that is likely to occur in vivo. Anthocyanins (delphinidin, cyanidin, and malvidin-3-glucosides) were incubated with phase II enzymes in the presence of activated cofactors in order to obtain glutathionyl conjugates, methylated and glucuronydated compounds. Overall, the three anthocyanins tested were metabolized in vitro. Two compounds were detected by HPLC after incubation of human liver cytosolic fraction with cyanidin-3-glucoside and one compound with delphinidin-3-glucoside. These compounds were identified as monomethylated products. LC-MS analysis yielded mass data that fit with the anthocyanin structures bearing an additional methyl group in ring B. Several compounds were detected by HPLC after incubation of human liver microsomes with malvidin, cyanidin, and delphinidin-3-glucosides. These compounds were identified as monoglucuronides products after HPLC analysis. Conjugation with glutathione also occurred as proved by the mass data obtained. However, in this case, two anthocyanin equilibrium forms (flavylium and chalcone or water adducts) conjugated with glutathione were detected. Overall, the data of the present work shows the feasibility of the in vitro enzymatic hemisynthesis of metabolites and glutathione conjugates of anthocyanins. This first experimental approach may further allow the achievement of new purified forms of anthocyanins, some of which do not occur in nature, and also the determination of whether these compounds are the bioactive forms responsible for some of the biological activities reported for anthocyanins.

KEYWORDS: Anthocyanins; metabolites; glutathione conjugates; glucuronydation; methylation

INTRODUCTION

Flavonoids have potential effects in cancer prevention and other health benefits; however, it has become clear that their bioactive forms in vivo are not necessarily those which occur in nature, but conjugates or metabolites arising from them after absorption (*1*). In particular, there is now strong evidence for the extensive phase I deglycosylation and phase II metabolism of the resulting aglycones to glucuronides and O-methylated forms during transfer across the small intestine and again in the liver (*2*). Thus, these structural modifications may provide a route for the bioavailability of dietary polyphenols to enable their beneficial effects. Many studies have been conducted with some flavonoids, such as (+)-catechin, (−)-epicatechin, (−)-epigallocatechin-3-gallate, (−)-epicatechin-3-gallate, resveratrol, and quercetin (*3*−*5*); however, very little progress has been made in establishing the pharmacokinetics of anthocyanins, with aspects such as absorption, biotransformation, and metabolism left essentially unstudied.

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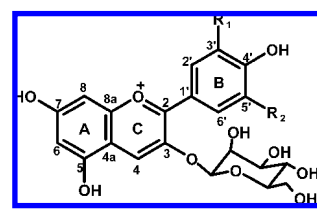
Anthocyanins are the most important group of water soluble plant pigments visible to the human eye. These compounds are natural food colorants, widely found in plant derived foodstuffs, such as berries, red grapes, cabbages, and other pigmented foods, plants, or vegetables (*6*, *7*). Depending on pH and the presence of chelating metal ions, anthocyanins are intensely colored blue, violet, or red. The structural differences between individual anthocyanins are related to the number of hydroxyl and methoxyl groups, the nature and number of sugars, and the position of these attachments in the aglycone (**Figure 1**) (*8*).

Depending on the nutrition habits, the daily intake of anthocyanins in humans has been estimated to be from 3 to 15 (*9*, *10*) up to 150 mg/day (*11*). The moderate consumption



Anthocyanin	R_1	R_2
Dp-3-gluc	OH	OH
Cy-3-gluc	OH	H
Pt-3-gluc	OCH_3	OH
Pn-3-gluc	OCH_3	H
Mv-3-gluc	OCH_3	OCH_3

Figure 1. Structures of *Vitis vinifera* anthocyanidin monoglucosides (flavylium form).

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Table 1. LC-MS Analysis of the Anthocyanin Metabolites

reaction	compound	M^+ (*m*/*z*)	$[M + H]^+$ (*m*/*z*)	MS^2 (*m*/*z*)	MS^3 (*m*/*z*)
methylation					
	CH_3-Dp-3-gluc	479		317 (−162)	303 (−14)
	CH_3−Cy-3-gluc	463		301 (−162)	287 (−14)
glucuronidation					
	$C_6H_8O_6$-Dp-3-gluc	641		303 (−338), 479 (−162)	
	$C_6H_8O_6$−Cy-3-gluc	625		287 (−338), 449 (−176), 463 (−162)	287 (−176)
	$C_6H_8O_6$-Mv-3-gluc	669		331 (−338), 493 (−176), 507 (−162)	331 (−176)
glutathione conjugates					
	SG-Cy-3-gluc	754			
	SG-Cy-3-gluc		772	463 (−309), 610 (−162), 754 (−18)	
	SG-Dp-3-gluc	770		479 (−291), 608 (−162)	335 (−144), 353 (−126), 461 (−18)
	SG-Dp-3-gluc		788	479 (−309), 608 (−162, −18), 626 (−162)	335 (−144), 353 (−126), 461 (−18)

of such compounds through the intake of products such as red wine (*12*) or bilberry extract (*13*) was described to be associated with the prevention of diseases related to oxidative stress, such as coronary heart diseases and cancer (*14*).

Studies on the biological activities demonstrated that anthocyanins and anthocyanidins are powerful antioxidants in various test systems (*15*−*17*). Furthermore, anti-inflammatory actions as well as antimicrobial and antitumor activities have been attributed to anthocyanins (*14*, *18*). So far, most of the research on the biological activities of anthocyanins with regard to their potential health effects has been performed in vitro. For that reason, some research in the past few years have focused on the bioavailability and biotransformation of anthocyanins, which could be major determinants of the biological activity of these compounds in vivo.

It has been previously reported that anthocyanins are poorly absorbed and circulate in the blood exclusively as unmetabolized parent glycosides (*19*−*21*). It is only recently that researchers have begun to suggest that anthocyanins are metabolized; however, the identification of derived metabolites has been limited as a result of their diversity and low concentrations in the blood. Anthocyanins are rapidly absorbed from both the stomach (*22*) and small intestine (*23*). They then appear in blood circulation and urine as intact, methylated, glucurono- and/or sulfoconjugated forms (*24*−*26*).

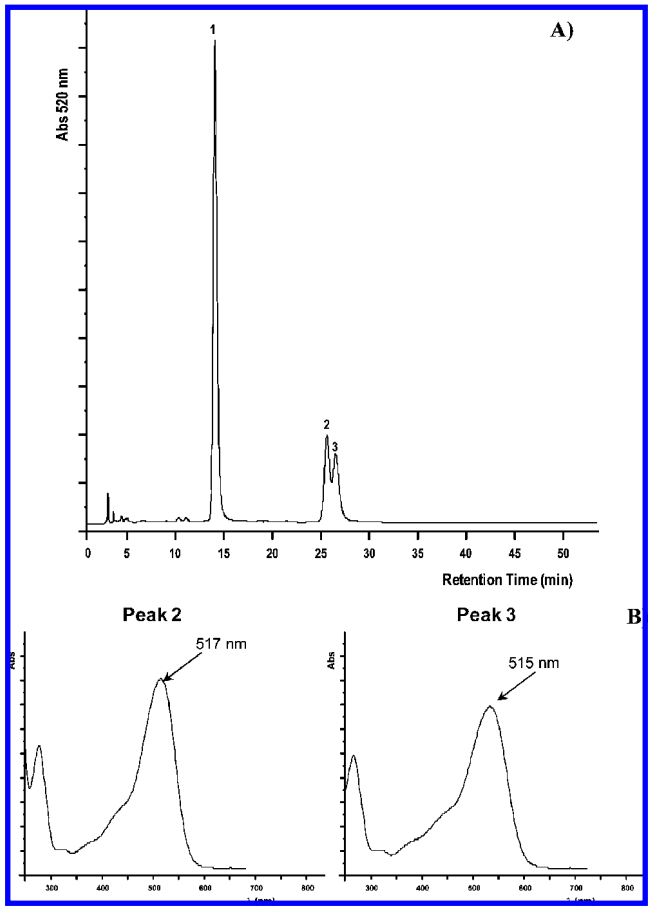

Figure 2. (**A**) HPLC chromatogram of the methylation products (2 and 3) of cyanidin-3-glucoside (1) recorded at 520 nm. (**B**) UV−vis spectra of the methylated products as recorded by the diode array detector.

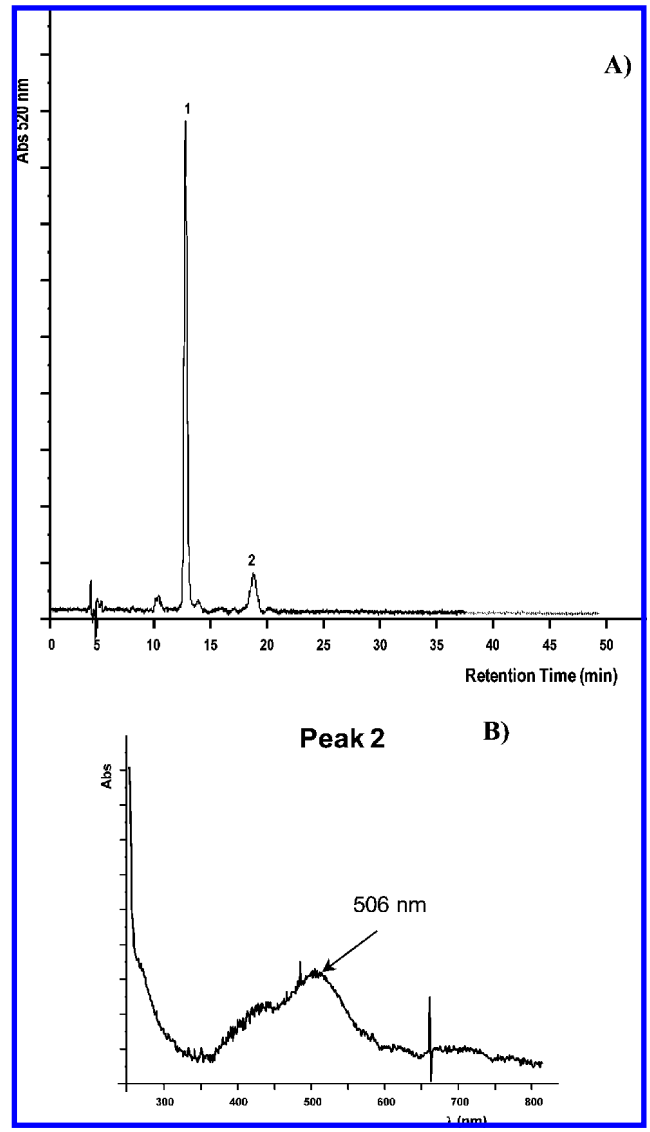

Figure 3. (**A**) HPLC chromatogram of the methylation product (2) of delphinidin-3-glucoside (1) recorded at 520 nm. (**B**) UV−vis spectrum of the methylated product as recorded by the diode array detector.

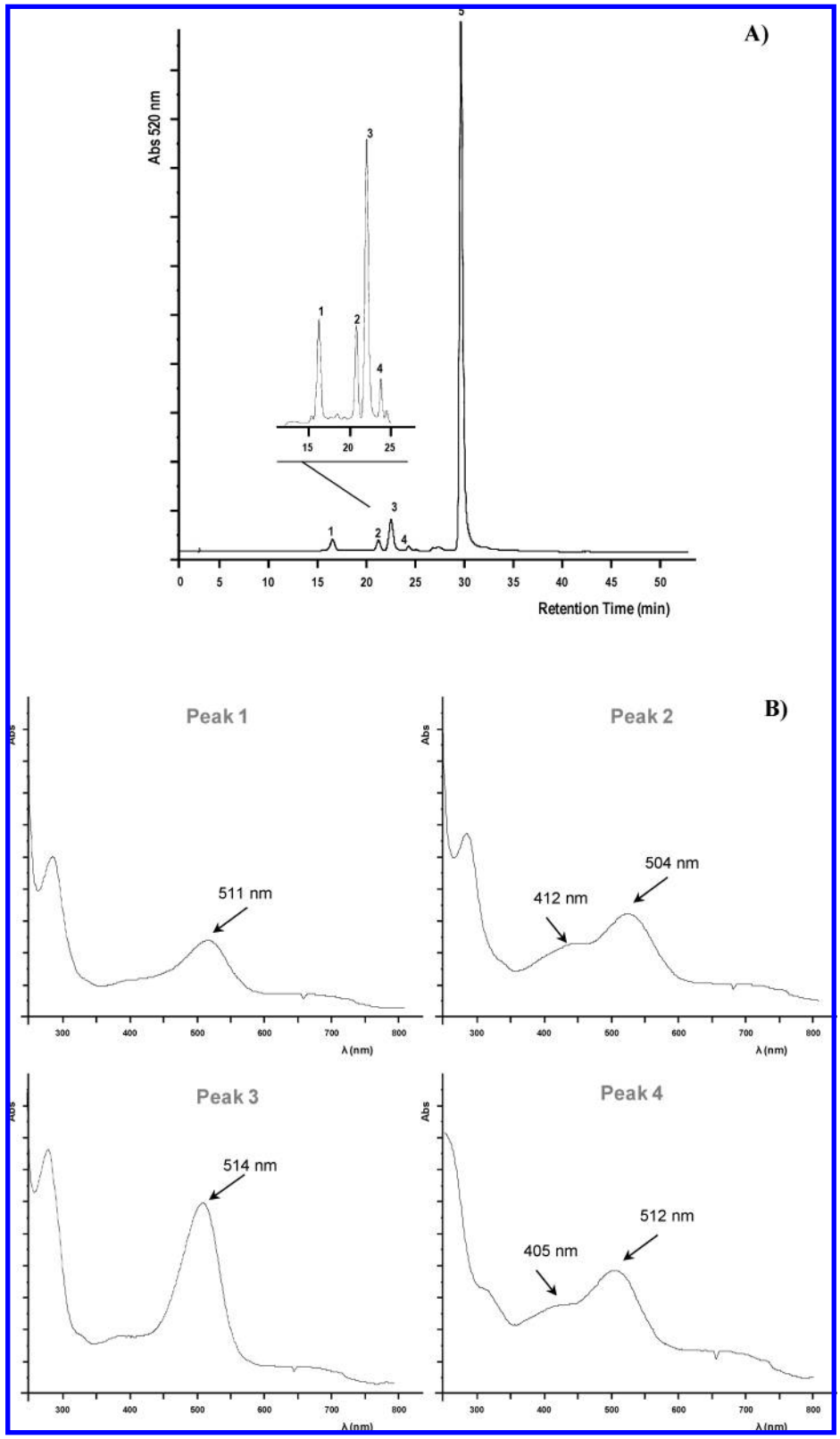

Figure 4. (**A**) HPLC chromatogram of cyanidin-3-glucoside (5) and its glucuronidated products (1−4), recorded at 520 nm. (**B**) UV−vis spectra of the glucuronidated products as recorded by the diode array detector.

The gut microflora seem to play an important role in the biotransformation of anthocyanins in the corresponding phenolic acids derived from the B-ring of the anthocyanin skeleton (*27*). As it is fundamental to determine whether these new compounds are the bioactive forms responsible for some of the biological activities reported for anthocyanins, the achievement of purified metabolites of anthocyanins is crucial. Bearing this, the in vitro phase II enzymatic hemisynthesis of new anthocyanin derived compounds was attempted. For that purpose, glycosides of cyanidin (Cy) and delphinidin (Dp), which represent the most abundant anthocyanins in fruits, and glycosides of malvidin (Mv) were extracted from grape skins *Vitis vinifera* and incubated with rat liver microsomes/cytosol (catechol-*O*-methyltransferases (COMT) and UDP-glucuronyltransferases (UGT)) or comercially available

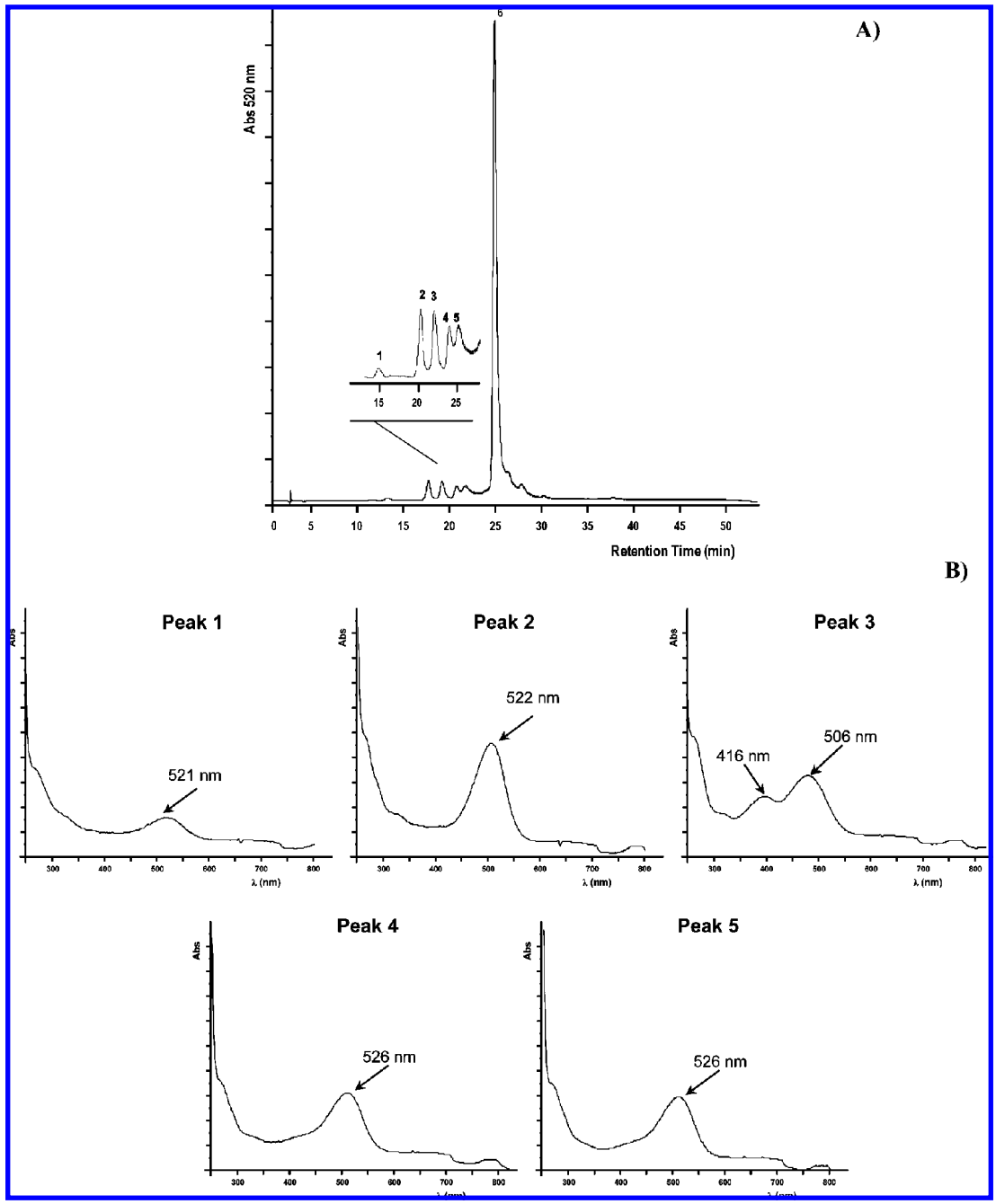

Figure 5. (**A**) HPLC chromatogram of delphinidin-3-glucoside (6) and its glucuronidated products (1−5), recorded at 520 nm. (**B**) UV−vis spectra of the glucuronidated products as recorded by the diode array detector.

enzymes (glutathione peroxidase (GP)). The analysis of the reaction samples was carried out by HPLC-DAD and LC-MS.

MATERIALS AND METHODS

Reagents. Toyopearl gel was purchased from Tosoh (Tokyo, Japan). Acetonitrile (CH_3CN) and dimethyl sulfoxide (DMSO) were purchased from Fluka (Madrid, Spain). Trichloroacetic acid (CCl_3COOH) was obtained from Merck (Darmstadt, Germany). *S*-Adenosyl-L-methionine (SAM), uridine 5′-diphosphoglucuronic acid (UDPGA), glutathione (GSH), glutathione peroxidase (GP), methanol (MeOH), hydrogen peroxide (H_2O_2), and formic acid (HCOOH) were obtained from Sigma-Aldrich (St. Louis, Missouri).

Anthocyanin Isolation. Grape skin anthocyanins (*Vitis vinifera*) were extracted with an aqueous solution of methanol (1:1) acidified with HCl, for 2 days at room temperature. The *Vitis vinifera* grape anthocyanin extract was filtered in a 50 μm nylon membrane and then purified by TSK Toyopearl gel column (250 × 16 mm i.d.) chromatography according to the procedure described previously (*28*). The extract was freeze-dried and stored at −18 °C until used.

The extract was analyzed by HPLC (Knauer K-1001) on a reversed-phase column (250 × 4.6 mm, 5 μm, C18) (Merck, Darmstadt); detection was carried out at 520 nm using a diode array detector (Knauer K-2800) as previously reported (*28*).

LC-MS analysis was performed on a liquid chromatograph (Hewlett-Packard 1100 series) equipped with an AQUATM (Phenomenex, Torance, CA, USA) reversed-phase column (150 × 4.6 mm, 5 μm, C18), thermostatted at 35 °C as reported elsewere (*28*).

HPLC Preparative Conditions. The pigments were purified by preparative HPLC (Knauer K-1001) on a reversed-phase column (250 × 25 mm, 10 μm, C18) (Merck, Darmstadt), and detection was carried out at 520 using a UV−vis detector (Hitachi, L-2420) (Merck, Darmstadt). The solvents were A, H_2O/HCOOH (9:1), and

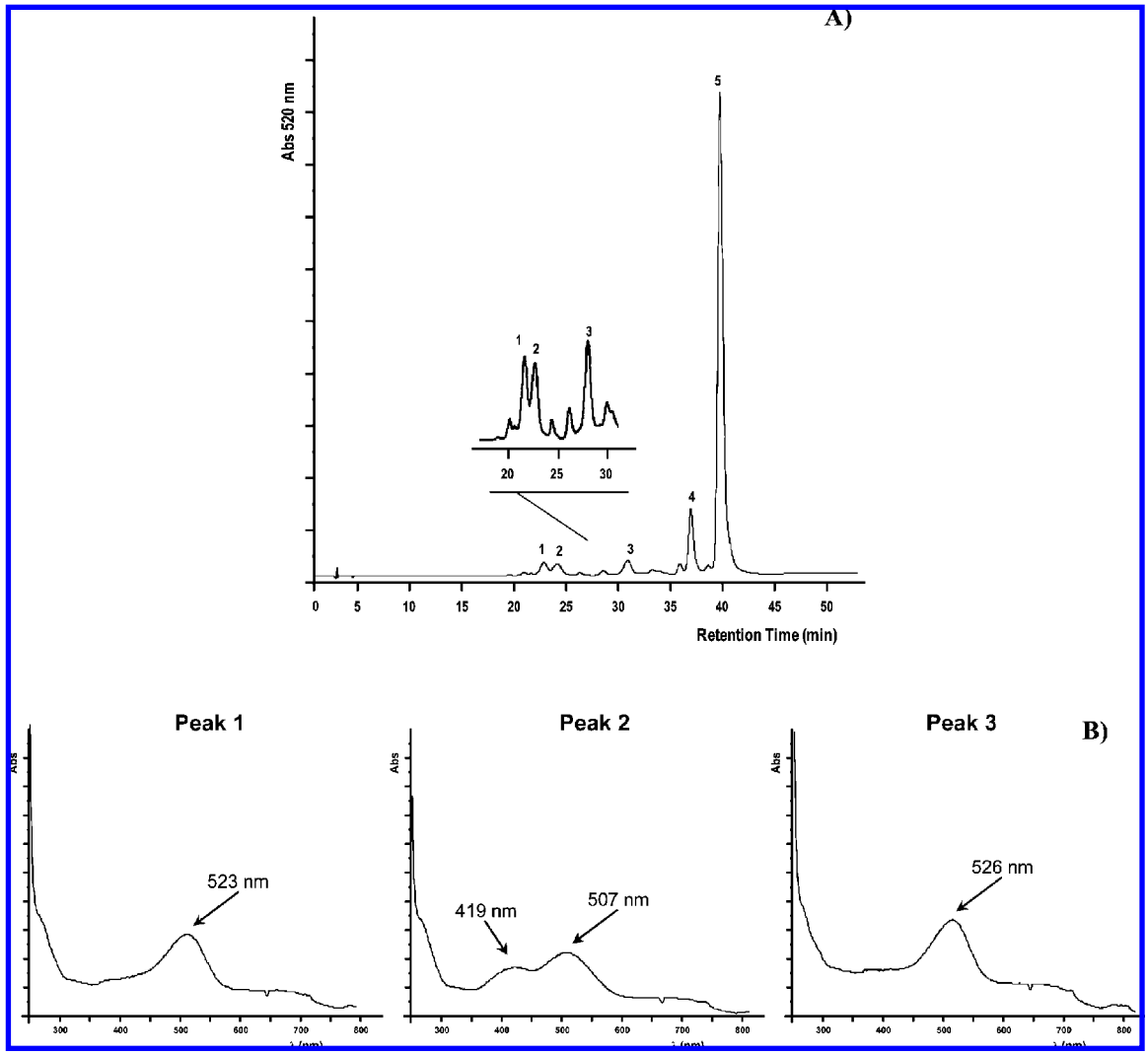

Figure 6. (**A**) HPLC chromatogram of malvidin-3-glucoside (5) and its glucuronidated products (1−3), recorded at 520 nm, peonidin-3-glucoside (4). (**B**) UV−vis spectra of the glucuronidated products as recorded by the diode array detector.

B, H_2O/MeOH/HCOOH (4:5:1). The gradient consisted of 65−15% A for 70 min at a flow rate of 10 mL min^{-1}. The column was washed with 100% B for 20 min and then stabilized at the initial conditions for another 20 min. Each pigment was collected and concentrated under vacuum. The purity of the isolated compounds was confirmed by HPLC-DAD-MS. The following anthocyanins were isolated: delphinidin-3-glucoside (Dp-3-gluc), cyanidin-3-glucoside (Cy-3-gluc), and malvidin-3-glucoside (Mv-3-gluc), although the last one was slightly contaminated with peonidin-3-glucoside.

Protein Extraction. Rats were anesthetized with sodium pentobarbital (300 mg/kg b.w.), and transcardiac perfusion was performed with ice-cold NaCl 0.9% (p/v) solution. Rat liver was extracted, weighed, and homogenized using a Thomas Teflon (DuPont, Wilmington, DE) homogenizer in solution A (62.5 mM KH_2PO_4, 50 mM Na_2HPO_4, and 0.1%Triton X-100, pH 7.4) (2 mL g^{-1}) and kept continuously on ice. This mixture was centrifuged at 12 000*g* for 20 min at 4 °C. The cytosolic supernatant was stored for further protein quantification. The resultant pellet was mixed with solution B (100 mM Hepes and 100 mM sacarose, pH 7.2) to form microsomes and centrifuged at 100 000*g* for 1 h at 4 °C (*29*). The microsomal pellet was washed in the same solution and subjected to a second centrifugation at 100 000*g* for 30 min. Finally, the microsomes were sonicated in solution B.

Protein Determination. The protein content of cytocolic and microssomal fractions were determined as described by Bradford (*30*), with bovine serum albumin as standard. The resulting cytosolic and microsomal fractions were divided into aliquots and frozen at −80 °C until use.

Rat COMT Catalyzed O-Methylation of Catechol or Pyrogallol-Containing Anthocyanins. Dp-3-gluc, Cy-3-gluc and Mv-3-gluc were incubated with rat liver cytosol protein fraction in a final volume of 250 μL of 10 mM Tris-HCl buffer, pH 7.4.

A typical incubation contained 4 mg mL^{-1} of the cytosol protein, which was first mixed with 1.2 mM $MgCl_2$, 1 mM DTT, and 2.5 mM SAM, and finally 400 μM of the substrate dissolved in DMSO (final concentration 4%) was added and the mixture incubated at 37 °C for up to 30 min to allow product formation under constant mild shaking. Negative controls were carried out by omitting SAM.

The reaction was stopped after 30 min by adding methanol (50 μL) to the reaction mixture to precipitate proteins. After centrifugation (5 min at 7000*g*), the samples were analyzed using HPLC-DAD (injection volume 50 μL). The samples were stored at −18 °C until further analysis. For the identification of the metabolite peaks, HPLC-DAD and LC-DAD-MS (in positive ion mode) analysis was used.

Rat UGT Catalyzed Glucuronidation of Anthocyanins. Glucuronidation of anthocyanins was carried out as described elsewhere with slight modifications (*27*). Dp-3-gluc, Cy-3-gluc, and Mv-3-gluc were incubated with rat liver microsomes in a final volume of 250 μL of 10 mM sodium phosphate buffer, pH 7.4. A typical incubation contained 4 mg mL^{-1} of the microsomal protein, which was first mixed with 100 mM of $MgCl_2$ and 2 mM of UDPGA, and finally 400 μM of substrate dissolved in DMSO (final concentration 4%) was added and the mixture incubated at 37 °C for up to 30 min to allow product formation under constant mild shaking. Negative controls were carried out by omitting UDPGA. The reaction was stopped as described above for O-methylation.

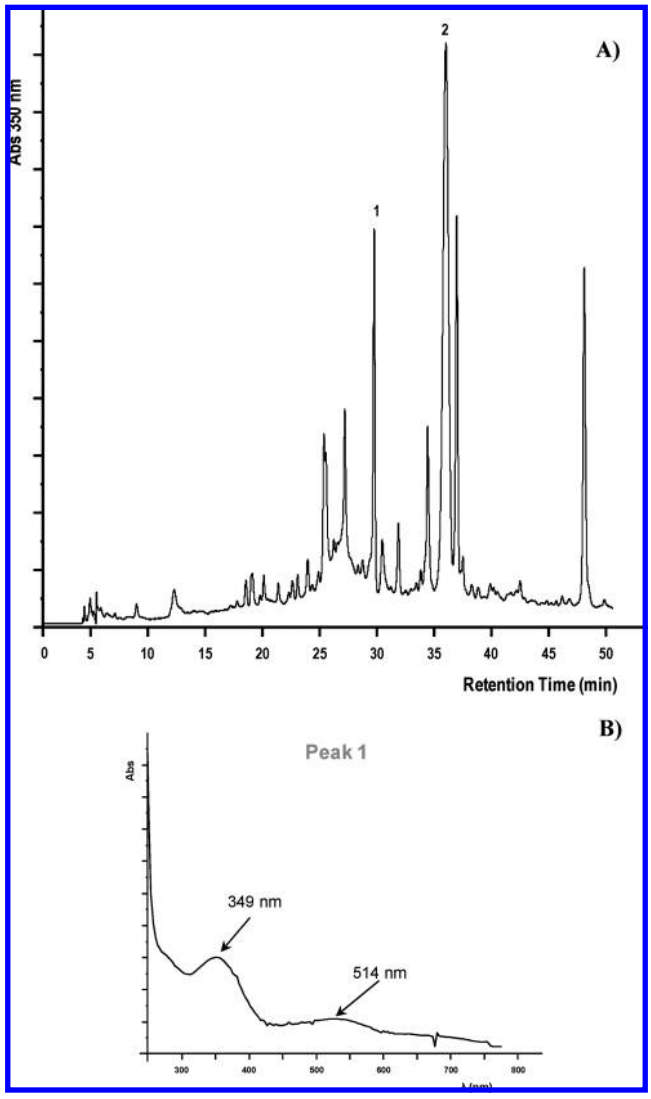

Figure 7. (**A**) HPLC chromatogram of cyanidin-3-glucoside (2) and its glutathionyl product (1), recorded at 350 nm. (**B**) UV−vis spectrum of the glutathionyl product as recorded by the diode array detector.

Oxidation of Anthocyanins by Horseradish Peroxidase in the Presence of Glutathione. Oxidation of anthocyanins by horseradish peroxidase in the presence of glutathione was carried out as described elsewhere for quercetin with slight modifications (*31*). Dp-3-gluc, Cy-3-gluc, and Mv-3-gluc, all added from a 10 mM stock solution in DMSO, were incubated with glutathione peroxidase in a final volume of 1000 μL of 50 mM sodium phosphate buffer, pH 7.0. A typical incubation contained 0.1 μM of glutathione peroxidase, which was first mixed with 1 mM of GSH and 150 μM substrate dissolved in DMSO (final concentration 1.5%). The reaction was started by the addition of 200 μM hydrogen peroxide.

The mixture was incubated at 37 °C for up to 30 min to allow product formation under constant mild shaking. Negative controls were carried out by omitting GSH.

The following additions were made to the incubation in the course of time for the generation of sufficient amounts of GSH conjugates of Dp-3-gluc, Cy-3-gluc, and Mv-3-gluc. Substrate (150 μM) was added every 5 min during the 30 min incubation period, 0.1 μM HRP was added at 0, 5, 15, and 20 min of incubation, and glutathione (1 mM) was added at 0 and 15 min of incubation. The reaction was stopped as described above for O-methylation.

HPLC-DAD Analysis. HPLC analysis of the anthocyanin methylated products was performed on an Elite Lachrom system (L-2130) equipped with a 250 × 4.6 mm i.d. reversed phase C18 column (Merck, Darmstadt); detection was carried out at 520 nm using a diode array detector (L-2455).

The solvents were A, H_2O/HCOOH (9:1), and B, H_2O/CH_3CN/HCOOH (6:3:1). The gradient consisted of 26−45% B for 50 min, 45−85% B for 25 min, and 85−0% B for 10 min at a flow rate of 1.0 mL min^{-1}. The column was washed with 100% B for 20 min and then stabilized at the initial conditions for another 20 min. Detected peaks were scanned between 200 and 600 nm. Compounds were first identified according to retention time and UV−vis spectra.

The anthocyanin glucuronidated and glutathione conjugated products were also analyzed by HPLC using the same conditions with a different gradient: 0−100% B for 70 min at a flow rate of 1.0 mL min^{-1}.

LC-MS Analysis. HPLC analysis of the anthocyanin methylated products was performed on a liquid chromatograph (Hewlett-Packard 1100 series) equipped with an AQUATM (Phenomenex, Torance, CA, USA) reversed-phase column (150 × 4.6 mm, 5 μm, C18), thermostatted at 35 °C. Solvents were A, H_2O/HCOOH (9.9:0.1), and B, H_2O/CH_3CN/HCOOH (6.9:3:0.1). The HPLC gradient used was the same as that reported above for the HPLC analysis. Double online detection was done in a photodiode spectrophotometer and by mass spectrometry. The mass detector was a Finnigan LCQ (Finnigan Corporation, San Jose, USA) equipped with an API source, using an electrospray ionization (ESI) interface. Both the auxiliary and the sheath gases were a mixture of nitrogen and helium. The capillary voltage was 3 V and the capillary temperature 190 °C. Spectra were recorded in positive ion mode between *m*/*z* 120 and 1500. The mass spectrometer was programmed to do a series of three scans: a full mass, a zoom scan of the most intense ion in the first scan, and a MS-MS of the most intense ion using relative collision energies of 30 and 60.

The anthocyanin glucuronidated and glutathione conjugated products were also analyzed by LC-MS using the same gradient reported above for the HPLC analysis.

RESULTS AND DISCUSSION

Delphinidin-3-glucoside (Dp-3-gluc), cyanidin-3-glucoside (Cy-3-gluc), and malvidin-3-glucoside (Mv-3-gluc) purified from grape skins (*Vitis vinifera*) (**Figure 1**) were incubated with rat liver microsomes, cytosol, or glutathione peroxidase as a source of phase II enzymes in the presence of activated cofactors in order to obtain glutathionyl conjugates, methylated and glucuronydated compounds. The metabolites obtained were analyzed by HPLC-DAD. In order to obtain structural information about the metabolites, all solutions were analyzed by LC-MS. The mass data of each metabolite and their fragments are summarized in **Table 1**.

Methylation. Dp-3-gluc, Cy-3-gluc, and Mv-3-gluc were incubated with rat liver cytosolic protein fraction as a source of phase II COMT enzyme in the presence of SAM.

In the case of the incubation of Cy-3-gluc, two new chromatographic peaks (2 and 3) with close retention times were detected in the HPLC chromatogram (**Figure 2A**). Analysis by LC-MS revealed two peaks at *m*/*z* 463, confirming the identity of these metabolites as monomethylated metabolites of Cy-3-gluc. Fragmentation of this ion yielded one peak at *m*/*z* 301, corresponding to the methylated aglycone. This observation indicates that the methylation site was not located on the sugar moiety. Attending to the enzyme specificity to the *ortho*-catechol group, the additional methyl group is supposed to be in ring B.

The analysis of the absorption spectra of the two methylated products (**Figure 2B**) allowed the identification of the position of the attached methyl groups. Metabolite 2 was assigned as peonidin-3-*O*-β-D-glucose from the comparison of the retention time, mass, and absorption spectra ($\lambda_{máx.}$ = 517 nm) of the pure standard in the conditions of analysis. Metabolite 3 was thus expected to have a methyl group at the C4′ position corresponding to the structure of isopeonidin-3-*O*-β-D-glucose. Cy-3-gluc has already been found, in vivo, metabolized in both the 4′ and 3′positions (*32*).

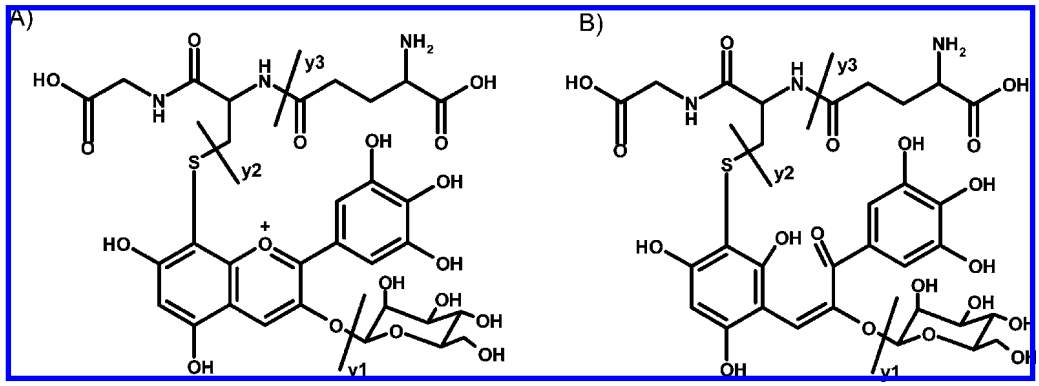

Figure 8. Fragmentation pattern of one Cy-3-gluc glutathionyl product in the form of flavylium (**A**) and chalcone (**B**) forms.

However, in the case of Dp-3-gluc, only one chromatographic peak was detected in the HPLC chromatogram (**Figure 3A**). MS fragments of the metabolite agreed with those of *O*-methyl delphinidin-3-glucoside (*m*/*z* 479) and its aglycone, *O*-methyl Dp (*m*/*z* 317) (**Table 1**), indicating that Dp-3-gluc was methylated in its aglycone moiety. However, the HPLC retention time of the metabolite was completely different from that of petunidin-3-*O*-*β*-D-glucose (Pt-3-gluc) (3′-*O*-methyl Dp-3-gluc), indicating that the site of methylation was likely to be the 4′-position of ring B. The UV−vis spectrum of the metabolite also supported the idea that the metabolite was not Pt-3-gluc ($\lambda_{máx}$ = 506 nm for *O*-methyl Dp-3-gluc and 540 nm for Pt-3-gluc) (**Figure 3B**). This observation is in accordance with what happens in vivo. This metabolite has also been identified in several tissues as a major metabolite, especially in the liver (*33*), but no 3′ or 5′-*O*-methyl Dp-3-gluc were detected. Therefore, 4′-OH methylation is the main path of Dp-3-gluc metabolism in rats (*33*). Furthermore, 4-*O*-methyl gallic acid, which is the corresponding phenolic acid that probably results from the cleavage of *O*-methyl Dp-3-gluc by intestinal microflora, has been detected in human urine (*34*, *35*).

These results are quite interesting because no naturally occurring food Dp-3-gluc or Cy-3-gluc methylated forms at the 4′ position of ring B have been identified. This work has thus shown that phase II metabolic modification of ingested anthocyanins can give rise to new anthocyanins that cannot be obtained from food sources. The anthocyanin Mv-3-gluc was also incubated with rat liver cytosol in the presence of SAM, but no methylated products were detected.

Glucuronidation. Another reaction in competition with the intestinal and/or chemical degradation at neutral pH in the gut is phase II glucuronidation, which could take place in the gut or, after absorption of the aglycone or the intact glucoside, in the liver. Therefore, it was assumed that the phase II glucuronidation mainly takes place in the liver and intestinal wall. The reaction of each anthocyanin with a system consisting of microsomes and UDPGA was used as an in vitro model for the glucuronidation reaction.

After incubation of Cy-3-gluc with rat liver microsomes in the presence of UDPGA, four newly formed compounds were detected by HPLC (**Figure 4A**). These compounds were identified as anthocyanin−monoglucuronides by LC-MS (**Table 1**). MS^2 fragments of the metabolites (*m*/*z* 625) were *m*/*z* 287 (−338), 449 (−176), and 463 (−162), indicating that Cy-3-gluc was glucuronidated in its aglycone moiety. The formation of four different monoglucuronides shows that glucuronidation takes place at any free hydroxyl position in the Cy molecule, namely, the hydroxyl positions at C5 and C7 of ring A and the hydroxyl groups at C3′ and C4′ of ring B.

This information is also important for the interpretation of the UV−vis spectra of the four monoglucuronide of Cy-3-gluc, which are shown in **Figure 4B**. Two of the monoglucuronides possess only one maximum of absorption, whereas the other two possess one clear maximum of absorption plus a marked shoulder peak around 410 nm. According to the literature, the preferred location for a second sugar would be at position 5 rather than at 7 or 4′ (*36*). Diglycoside anthocyanins present absorption maxima toward lower wavelengths than the corresponding monoglycosides, and the presence of a shoulder in the visible region at about 440 nm in the 3-monoglycosides is not seen in the 3,5-diglycosides. A shoulder has been observed in the UV−vis spectra of Cy and Dp derivatives with *O*-glycosyl moieties on their B-rings (*37*, *38*). Another work reported that the UV−vis spectra of anthocyanidins with a sugar moiety in the 5-position of ring A do not display the shoulder peak around 420−440 nm (*39*), which could help to explain the observed results herein. Therefore, it can be proposed that Cy-3-gluc-monoglucuronides 1 and 3 are glucuronidated at ring A, whereas Cy-3-gluc-monoglucuronides 2 and 4 are ring B glucuronides.

Concerning the incubation of Dp-3-gluc, five Dp-3-gluc-monoglucuronides were identified by HPLC-DAD-MS analysis (**Figure 5A**). Only one glucuronidated group is present in each new compound as all displayed the same mass of *m*/*z* 641, which is characteristic of the Dp-3-gluc monoglucuronide. Fragmentation of this ion by LC-MS yielded two peaks at *m*/*z* 303 and 479, corresponding to the loss of glucose and glucuronyl plus glucose, respectively (**Table 1**). The fragmentation pattern indicates that the glucuronidation site was located on ring A or B.

To clarify the glucuronidation site of the metabolites, UV−vis spectra of each peak was analyzed and compared with those of authentic anthocyanin (**Figure 5B**).

The analysis of the UV−vis spectra of the five glucuronidated products revealed maximum absorption wavelengths obtained for peaks 1, 2, 3, 4, and 5 at 521, 522, 506, 526, and 526 nm, respectively. As seen in **Figure 5B**, only peak 3 possesses one clear maximum absorption plus a marked shoulder peak around 415 nm, whereas the other monoglucuronides possessed only one absorption maximum.

According to the former argument, it was expected that only two of the detected peaks lacked the presence of a shoulder in the visible region at about 440 nm. However, in the experimental conditions used and in result of the minor quantities of metabolites obtained, as seen in **Figure 5B**, they could not be

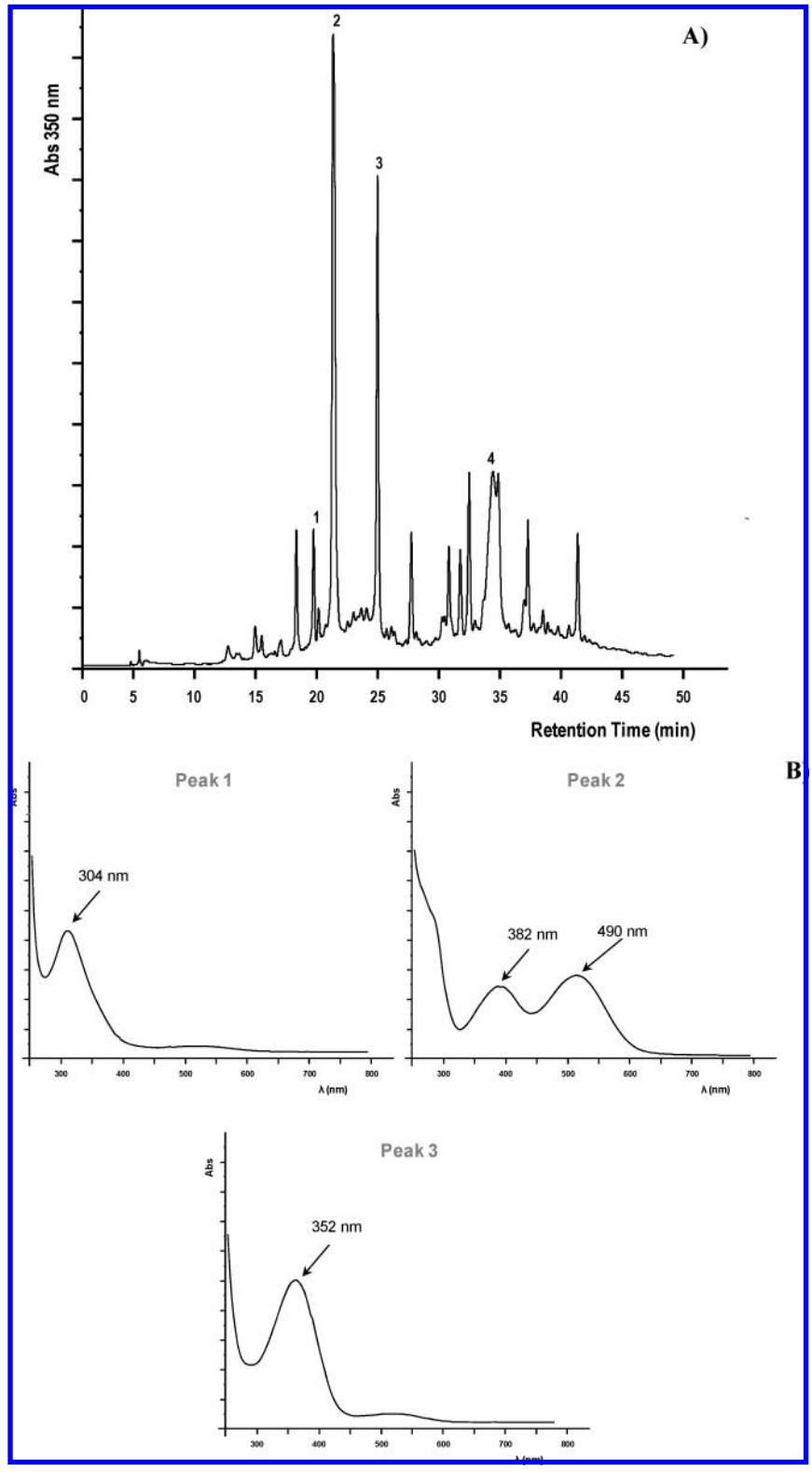

Figure 9. (**A**) HPLC chromatogram of delphinidin-3-glucoside (4) and its glutathionyl products (1−3), recorded at 350 nm. (**B**) UV−vis spectra of the glutathionyl products as recorded by the diode array detector.

detected. Therefore, it is only possible to assume that peak 3 does not correspond to Dp-3-gluc glucuronidated in ring A.

In the case of Mv-3-gluc, three different glucuronidated products were detected by HPLC-DAD (**Figure 6A**) corresponding to the substitution of each of the OH free groups in the positions C4′, C5, and C7. These peaks were identified as monoglucuronides by HPLC-MS (**Table 1**). As previously reported, a sugar substitution in ring A results in a single peak in the UV−vis spectra that corresponds to the maximum wavelength without any shoulder.

It can thus be assumed that the substitution in position C4′ should correspond to metabolite 2, as seen from the respective UV−vis spectrum (**Figure 6B**).

Additionally, hypsochromic shifts at maximum wavelengths observed in the spectra of some conjugates tentatively identified

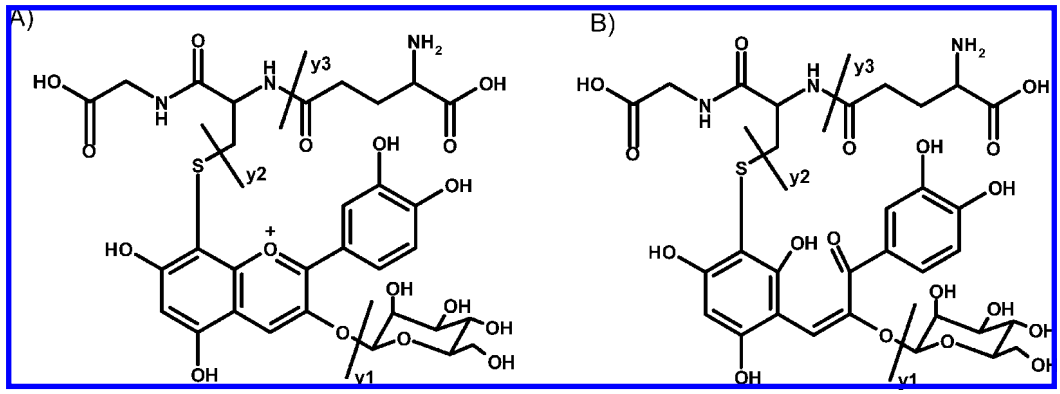

Figure 10. Fragmentation pattern of one Dp-3-gluc glutathionyl product in the form of flavylium (**A**) and chalcone (**B**).

as glucuronidated in ring B were also found for anthocyanidins glycosylated in the B-ring (*38*).

Interestingly, the anthocyanidin monoglucosides were all substrates for UGTs. Curiously, once again no product with more than one substitution was detected.

Glutathione Conjugates. Previous investigations with chemical systems have shown that quercetin (*40*, *41*) and other catechol-containing flavonoids, such as catechin (*42*), taxifolin, luteolin (*43*), fisetin, and 3,3′,4′-trihydroxyflavone (*44*), react with GSH to generate mono- and diglutathionyl adducts. Because the structural similarities between these compounds and anthocyanins are expected, the same adduct formation occurs using similar experimental conditions.

The HPLC chromatogram recorded at 350 nm, after the incubations of Cy-3-gluc with GP/H_2O_2 in the presence of glutathione, is shown in **Figure 7A**.

LC-MS data, have shown a peak at *m*/*z* 772 (peak 1), which fits with the structure of Cy-3-gluc glutathionyl adducts linked with one water molecule, probably yielding the chalcone form of the anthocyanin moiety (**Table 1**). The MS^2 fragment pattern of peak 1 showed fragments at *m*/*z* 754 (−18) corresponding to the loss of one water molecule, at *m*/*z* 610 (−162) corresponding to the loss of one glucose molecule, and at *m*/*z* 463 (−18, −162, and −129) that may correspond to the two previous losses plus a fragment of the glutathione molecule, probably resulting from fragmentation y3 (**Figure 8B**). The UV−vis spectrum of this compound 1 showed one maximum absorption at 350 nm characteristic of the chalcone form of anthocyanins (**Figure 7B**).

Traces of the compound detected at *m*/*z* 754 probably corresponding to Cy-3-gluc glutathionyl adduct were also detected by LC-MS between 25 and 28 min.

The conjugation of glutathione with 3′,4′-dihydroxy flavonoids has been shown to be pH-dependent in cell-free systems. Effectively, it has been demonstrated that low pH favored the formation of ring A conjugates, while less acidic conditions led to the generation of ring B adducts (*40*). Thus, in Cy-3-gluc, ring A is likely to be the site of glutathione adduct formation with anthocyanin.

The HPLC chromatogram recorded at 350 nm, after incubation of Dp-3-gluc with GP/H_2O_2 in the presence of glutathione showed three new chromatographic peaks (1, 2, and 3) with close retention times (**Figure 9A**).

The mass data have shown a peak at *m*/*z* 770 (peak 2), which fits with the structure of the Dp-3-gluc glutathionyl adduct. Peaks 1 and 3 at *m*/*z* 788 possibly match the Dp-3-gluc glutathionyl adducts bearing one water molecule, corresponding to the chalcone form of anthocyanin (**Table 1**).

MS^2 fragments of peak 2 originated fragments at *m*/*z* 608 (−162) that may correspond to the loss of one glucose molecule and another at *m*/*z* 479 (−162 and −129) that may correspond to the loss of one glucose molecule and a fragment of the glutathione molecule, probably resulting from fragmentation y3 (**Figure 10A**). MS^2 fragment patterns of peaks 1 and 3 showed fragments at *m*/*z* 754 (−18) corresponding to the loss of one water, at *m*/*z* 610 (−162) corresponding to the loss of one glucose molecule, and at *m*/*z* 479 (−18, −162, and −129) that may correspond to the two previous losses plus a fragment of the glutathione molecule, probably resulting from fragmentation y3 (**Figure 10B**).

Some quercetin glutathionyl adducts were already described with a similar fragmentation pattern (*45*).

The UV−vis spectra of Dp-3-gluc glutathionyl adducts indicated that peaks 1 and 3 possess one maximum absorption at 304 and 350 nm, respectively, while peak 2 possesses one maximum around 490 nm and a shoulder around 380 nm (**Figure 9B**). These absorption maxima may indicate that two anthocyanin equilibrium forms, the flavylium (peak 2) and the respective chalcone form of anthocyanins (peaks 1 and 3), are present.

Although peaks 1 and 3 showed the same mass value and fragmentation pattern, they had different retention times, 17.96 and 22.94 min. The different global polarity and spectroscopic characteristics of the newly formed compounds thus indicate that the substitutions may have occurred in different positions of the anthocyanin. The most probable sites are the C6 and C8 positions of ring A, as the formation of ring A conjugates is favored by acidic reaction conditions. For quercetin, two ring A monoglutathione conjugates (8-glutathionyl quercetin and 6-glutathionyl quercetin), at pH 7.0, have recently been observed (*41*). In the case of Mv-3-gluc, no glutathionyl adducts were detected.

More studies are needed for structural identification of the products detected in order to better understand the molecular pathways behind glutathionyl conjugate formation.

Conclusions. The present work may provide not only important purified metabolites for the evaluation of biological effects of anthocyanin metabolites in comparison with their precursors but also standards for the following identification of anthocyanin metabolites in biological samples. The in vitro hemisynthesis reactions described herein are expected to mimic the in vivo situations and could help to better understand the absorption, bioavailability, and metabolism of these compounds.

Overall, where anthocyanins are concerned, a final and complete evaluation of the biological effects of anthocyanins will have to take all metabolites and degradation products of these compounds into account. When considering the possible bioactive mechanisms of action of anthocyanins and their in vivo metabolites in cell systems, it is important to consider their uptake and possible further metabolization by the cells. The

question is raised whether the anthocyanins themselves or their metabolites are responsible for the reported biological effects. Further investigations are necessary to elucidate the precise mechanisms of action of these compounds.

ABBREVIATIONS USED

Dp-3-gluc, delphinidin-3-glucose; Cy-3-gluc, cyanidin-3-glucose; Mv-3-gluc, malvidin-3-glucose; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography−mass spectrometry; COMT, catechol-*O*-methyltransferases; UGT, UDP-glucuronyltransferases; GP, glutathione peroxidase.

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