Isolation of Flavanol-Anthocyanin Adducts by Countercurrent Chromatography

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Abstract

Pigments of the flavanol-anthocyanin (F-A$^+$) type detected earlier in wine are synthesized using a protocol adapted from the synthesis of procyanidin dimers. The F-A$^+$ adduct thus obtained is purified by countercurrent liquid–liquid partition, currently referred to as countercurrent chromatography (CCC). The solvent system consists of tert-butyl methyl ether–*n*-butanol–acetonitrile–water (2:2:1:5, acidified with 0.1% trifluoroacetyl) with the light organic phase acting as a stationary phase and the aqueous phase as the mobile phase. Four fractions are recovered and analyzed by high-performance liquid chromatography coupled to a diode-array detector and electrospray ionization mass spectrometer. The multilayer CCC method allowed the separation of pigments in three different groups. The first group consists of hydrosoluble pigments present in fraction 1; the second group consists of the F-A$^+$ adducts [catechin–malvidin 3 glucoside (Mv3glc), along with some (catechin)$_2$–Mv3glc]; and the third group is their anthocyanin precursor, Mv3glc.

Introduction

Red wine is a very complex medium, which evolves during its conservation and aging. With storage time, the color of young red wine changes from red-bluish towards the reddish-brown color of matured wines and the astringency decreases. These color changes are attributable to gradual conversion of the anthocyanin red grape pigments to various derivatives by different reaction mechanisms, including direct reactions between anthocyanins and flavanols. Two mechanisms, leading respectively to anthocyanin-flavanol (A$^+$-F) or flavanol-anthocyanin (F-A$^+$) (or both) pigments, are postulated for direct reactions between anthocyanins and flavanols. So far, only F-A$^+$ adducts have been observed in wine (1) and have recently been successfully synthesized (2).

In the formation of F-A$^+$, proanthocyanidins (F-F) are affected by acid-catalyzed cleavage of their interflavanyl bond, releasing the intermediate carbocation F$^+$ (3), which acts as an electrophile. Nucleophilic addition of the anthocyanin in its hydrated hemiketal form (AOH) (4) yields the colorless dimer (F-AOH), which dehydrates to the red flavylium form (F-A$^+$). Isolation of these pigments is essential to formally establish their structure, determine their chemical and color properties, and compare them with those of genuine anthocyanins.

As an alternative to the classical gel chromatographic techniques, countercurrent chromatography (CCC) has proven efficient in the preparative fractionation of anthocyanins (5,6). This method is a chromatographic separation process in which a liquid phase is retained in a coil by centrifugal force, while a second immiscible liquid phase continuously passes through it. Because it is a liquid–liquid partition technique in which no solid support is used, the retention of the solutes is determined exclusively by their partition coefficients and, equally important, the problem of their irreversible adsorption onto the stationary phase is excluded (7,8).

CCC has enabled the separation of native anthocyanins from derived anthocyanins in wine (5,9,10). The aim of this study was to separate the F-A$^+$ adduct obtained by synthesis from its anthocyanin precursor by means of CCC.

Experimental

Pigments of the F-A$^+$ type were synthesized by using a protocol adapted from the synthesis of procyanidin dimers (11), in which the terminal flavanol unit was replaced with malvidin 3 glucoside (Mv3glc) (2). The solution containing the F-A$^+$ adduct was freeze-dried and separated with a multilayer countercurrent chromatograph (MLCCC) manufactured by P.C. Inc. (Potomac, MD), equipped with a 380-mL coil column using the following conditions: the solvent system consisted of tert-butyl methyl ether–*n*-butanol–acetonitrile–water [2:2:1:5, acidified with 0.1% trifluoroacetyl (TFA)] with the light organic phase acting as the stationary phase and the aqueous phase as the mobile phase. The

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elution mode was head to tail (5). The rotation speed was 800 rpm and flow rate 0.8 mL/min. The sample was dissolved in 10 mL of a mixture (50:50, v/v) of the mobile and stationary phases before injection. Four different fractions and the organic stationary phase were recovered and freeze-dried. Solutions were analyzed by high-performance liquid chromatography (HPLC)–diode-array detection (DAD) and HPLC–electrospray ionization (ESI) mass spectrometry (MS).

Analysis of fractions by HPLC–DAD and HPLC–MS

HPLC–DAD

HPLC–DAD analyses were performed using a MD-910 multi-wavelength detector (220–650 nm) equipped with a DG-980-50 three-line degasser, LG-980-02 ternary gradient unit, PU-980 Intelligent HPLC pump, and Borwin photodiode-array detection (PDA) chromatography software (Jasco, Gross-Umstadt, Germany). The samples were injected via a Rheodyne 7125 injection valve (Techlab, Erkerode, Germany) equipped with a 20-µL loop, and separations were carried out on a Luna RP-18 column (250- × 4.6 mm-i.d., 5 µm) (Phenomenex, Aschaffenburg, Germany) at a flow rate 0.5 mL/min. Two solvent systems were used. Solvent A consisted of water–acetonitrile–formic acid (87:3:10, v/v/v), and solvent B consisted of water–acetonitrile–formic acid (40:50:10, v/v/v). The linear gradient was as follows: 0 min, 6% B; 20 min, 20% B; 35 min, 40% B; 40 min, 60% B; 45 min, 90% B; and 55 min, 6% B.

HPLC–ESI-MS

MS analysis and fragmentation experiments were performed on a ThermoFinnigan LCQ Advantage (San Jose, CA) MS equipped with an ESI source and an ion trap mass analyzer, which were controlled by the LCQ navigator software. The MS was operated in the positive ion mode in the range *m/z* 150–2000 and under the following conditions: source voltage, 4.5 kV; capillary voltage, 23.5 V; capillary temperature, 250°C; and collision energy for fragmentation, 25% for MS^2, 30% for MS^3, and 35% for MS^4. Chromatographic separation was achieved using a Waters 2690 system equipped with an autosampler system, Waters 996 PDA detector, and Millennium 32 chromatography manager software (Waters, Bedford, MA). The column was a Lichrospher 100-RP18 (250- × 2-mm i.d., 5-µm packing) protected with a guard column of the same material (Merck, Darmstadt, Germany). The elution conditions were as follows: flow rate, 0.250 mL/min; oven temperature, 30°C; solvent A, water–formic acid (95:5, v/v); and solvent B, acetonitrile–water–formic acid (80:15:5, v/v/v). Elution started isocratically with 2% B for 7 min, continued with linear gradient from 2% to 20% B in 15 min, from 20% to 30% B in 8 min, from 30% to 40% B in 10 min, from 40% to 50% B in 5 min, and from 50% to 80% B in 5 min, followed by washing and re-equilibration of the column.

Phloroglucinolysis

Acid-catalyzed cleavage in the presence of excess phloroglucinol was performed on fraction 1, according to the protocol developed by Kennedy and Jones (12). A solution of 50 g/L of phloroglucinol in methanol containing 0.1N HCl (no ascorbic acid was added) was prepared and added to fraction 1. The reaction lasted 20 min at 50°C. The solution was then analyzed by LC–DAD–MS, as described previously.

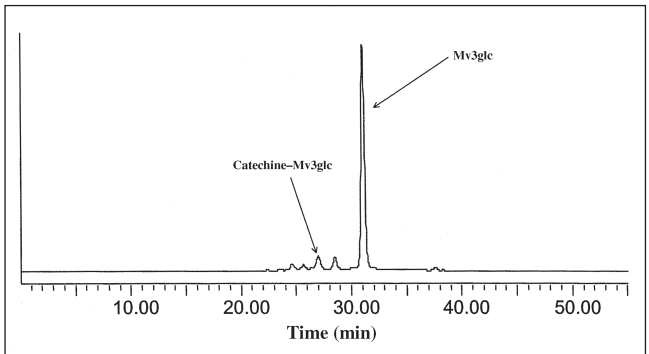

Figure 1. HPLC profile at 520 nm of the reaction media.

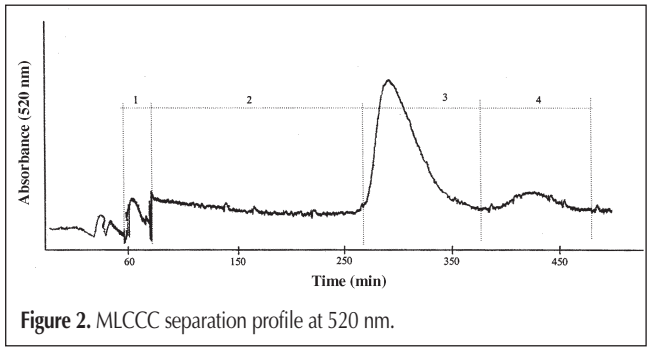

Figure 2. MLCCC separation profile at 520 nm.

Results and Discussion

Catechin–Mv3glc was synthesized, as described earlier (2), by a reaction involving nucleophilic addition of Mv3glc on the flavanol carbocation generated from taxifolin, following a protocol developed for procyanidin synthesis (11). Analysis of the resulting solution by HPLC–ESI-MS showed the presence of a pigment with a mass signal at *m/z* 781, in the positive ion mode. This ion was attributed to the expected catechin–Mv3glc dimer. Its characteristic fragmentation pattern obtained by the MS^2 and MS^3 experiments (loss of a 126 fragment corresponding to the A ring) confirmed that the flavanol was in the upper position linked at C4 (2).

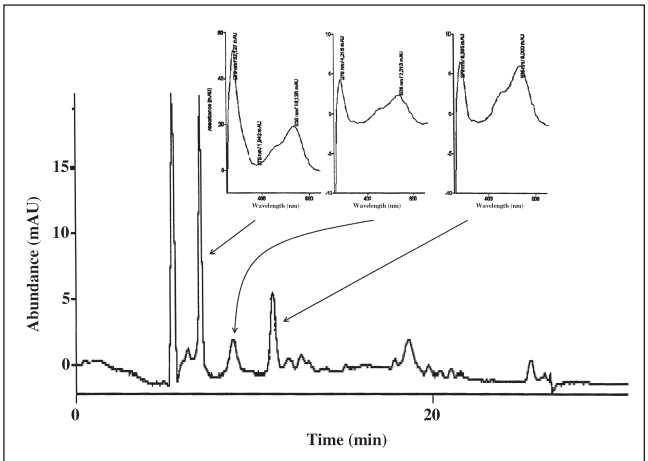

Figure 3. HPLC profile at 520 nm of fraction 1.

As can be seen in the HPLC profile at 520 nm (Figure 1), there was a large excess of unreacted Mv3glc in proportion to the catechin–Mv3glc dimer.

The MLCCC separation profile at 520 nm of the reaction mixture is shown in Figure 2. Four different fractions were noted 1 to 4, and the organic stationary phase was recovered and freeze-dried. The MLCCC separation lasted over 9 h.

Fraction 1 consisted of water-soluble pigments (Figure 3). HPLC analysis of this fraction showed the presence of a major peak eluting very early and with two absorption maxima at 278 and 535 nm, indicating that it contains a flavylium chromophore. The absorbance value at 280 nm greatly exceeded that at 520 nm, suggesting that it is actually a polymeric species in which each flavylium unit is associated to several flavanols (or anthocyanins under colorless forms). The peaks eluting next also showed a higher absorbance value at 280 nm than at 520 nm. These series of pigments showed a more polar behavior than Mv3glc and the catechin–Mv3glc adduct. The increase in polarity might reflect the higher percentage of nonflavylium units incorporated in these pigments. Larger molecular weight tannin–anthocyanin adducts have earlier been reported to be recovered in the aqueous phase after liquid–liquid extraction (1). Fraction 1 was submitted to acid-catalyzed cleavage in the presence of pholoroglucinol and anlayzed by HPLC–DAD–MS.

Acid-catalyzed cleavage in the presence of a nucleophilic agent such as phloroglucinol or toluene-α-thiol was initially developed for the analysis of proanthocyanidins (12–15) but can also provide structural information on tannin–anthocyanin derivatives (1,16,17). It relies upon acid-catalyzed depolymerization in the presence of phloroglucinol, used as a nucleophilic agent to trap the carbocations released by cleavage of the interflavanyl bonds. Analysis of the resulting solution then makes it possible to distinguish the terminal units (substituted at C-6 or C-8 in the original structure) that are released as such and the upper and intermediate units (substituted at the C-4 position in the original structure) that yield the corresponding phloroglucinol derivatives.

HPLC–DAD–MS analysis of fraction 1 after phloroglucinolysis

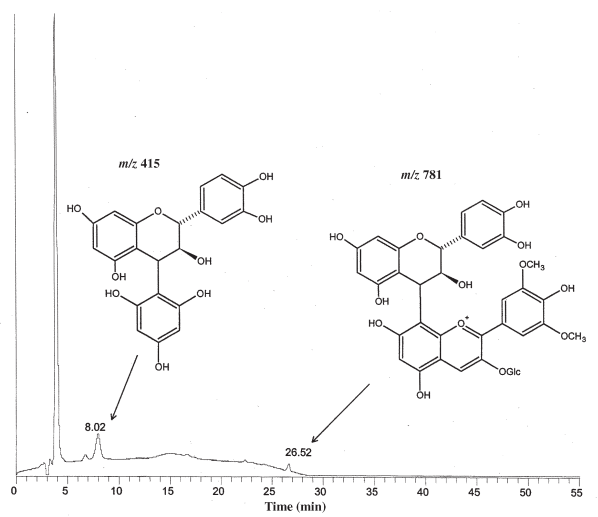

Figure 4. HPLC profile at 280 nm of fraction 1 after phloroglucinolysis.

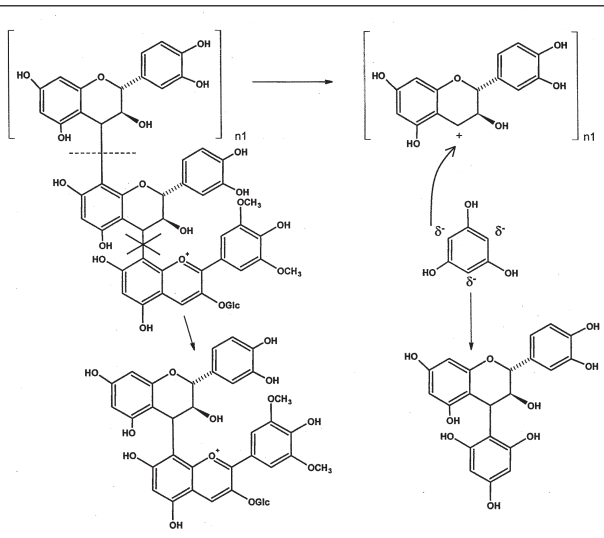

Figure 5. Mechanism of phloroglucinolysis on cat_n–anthocyanin adducts ($n > 1$).

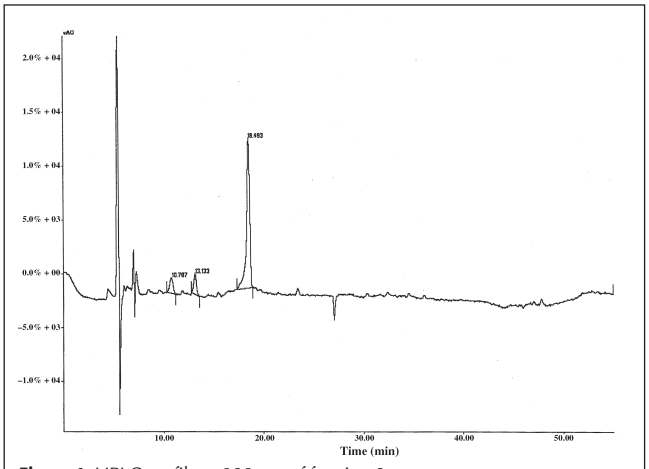

Figure 6. HPLC profile at 320 nm of fraction 2.

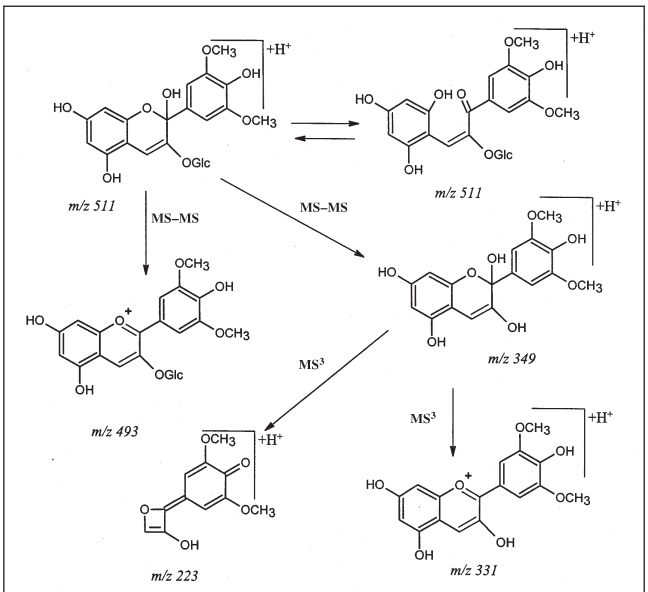

Figure 7. MS^2 and MS^3 analysis of the molecular ion at *m/z* 511.

showed the disappearance of the series of polar pigments shown in Figure 3. Analysis of this fraction also showed the presence of a pigment with the same retention time, UV–vis and mass spectra as the catechin–Mv3glc dimer, and product with a λ_{max} at 278 nm detected at *m/z* 415 with a fragment at *m/z* 289, identified as the phloroglucinol derivative of catechin (Figure 4).

The release the phloroglucinol derivative of catechin and of F-A$^+$ dimer after phloroglucinolysis of fraction 1 reflects the presence of cat$_n$-anthocyanin adducts ($n > 1$) before phloroglucinolysis (shown in Figure 5). The linkage in the terminal cat-anthocyanin residue has proven resistant to acid-catalyzed cleavage (2).

Fraction 2 did not present any absorbance at 520 nm (Figure 2). However, its HPLC profile at 320 nm (Figure 6) showed a major peak with a mass signal at *m/z* 513 and a λ_{max} at 310 nm. A compound with a λ_{max} at 280 nm detected at *m/z* 511 was assigned to Mv3glc in its hydrated form and was also present in fraction 2. MS^2 fragmentation of this ion (Figure 7) gave two signals at *m/z* 493 and 349, corresponding to the loss of water (–18 amu) and glucose (–162 amu). MS^3 fragmentation of the ion at 349 gave two signals at *m/z* 331 and 223 that correspond to the loss of water (–18 amu) and to the loss of 126 amu (A ring), as described for flavanols (18–19) and chalcone (20). The major peak, with a λ_{max} at 310 nm, was detected at *m/z* 513. Its MS^2 fragmentation (Figure 8) gave signals at *m/z* 495, 351, and 375, which correspond, respectively, to the loss of a water molecule (–18 amu), loss of the glucose moiety (–162 amu), and retro Diels-Alder (RDA) decomposition of the heterocycle (–138 amu). MS^3 fragmentation of the ion at *m/z* 495 gave a signal at *m/z* 333 that corresponds to the loss of glucose (–162 amu). MS^4 fragmentation of the ion at *m/z* 333 gave a signal at *m/z* 305 that corresponds to the loss of a CO (–28 amu) as has been observed for flavonol (21). MS^3 fragmentation of the ion at *m/z* 351 gave signals at *m/z* 225, 139, and 333 that correspond to the loss of the A ring (–126 amu), to the RDA decomposition of the heterocycle (–212 amu), and to the loss of water (–18 amu). MS^4 fragmentation of the ion at *m/z* 333 gave a signal at *m/z* 139 that corresponds to the RDA decomposition of the heterocycle (–194 amu). The mass signal at *m/z* 513 was thus interpreted as being the flavan form of Mv3glc hydroxylated in its C2 position. However, this proposed structure does not match with the detected λ_{max} at 310 nm. This absorbance could arise from another compound that does not respond to MS under our analytical conditions. The presence of the ions at *m/z* 511 and 513 in fraction 2 might be attributable to the synthesis procedure in which $NaBH_4$ is used to reduce taxifolin to a flavan-3,4-diol, followed by dehydration and protonation to issue the catechin$^+$ carbocation. The anthocyanin is then added as a mixture of hydrated and flavylium forms. The flavene form of Mv3glc may result from reduction by $NaBH_4$ of the anthocyanin flavylium. The ion at *m/z* 513 could arise from the addition of water on C-2 of Mv3glc under the flavene form (Figure 9), similar water addition has been observed for quercetin (22). The fragmentation patterns of the ions at *m/z* 511 and 513 showed the typical loss of the glucose moiety (–162 amu), confirming their anthocyanin nature. However, these fragmentation patterns are not "flavylium-like", given that anthocyanins under their flavylium form will break up in small fragments (23). These fragments are more "tannin-like" because there are typical flavanol fragmentations like RDA and A-ring loss, which had already been observed for anthocyanins, that are not under the flavylium (24,25).

Fraction 3 contained unreacted residual Mv3glc, which is the major anthocyanin in grapes and young wines, and trace amounts of delphinidin 3 glucoside and petunidin 3 glucoside, which were present in the Mv3glc sample used for the synthesis reaction.

Fraction 4 contained the F-A$^+$ oligomeric derivatives along with small amounts of residual Mv3glc (Figure 10). Before, MLCCC Mv3glc represented 95% of the peak area at 520 nm and the catechin–Mv3glc dimer represented 3%. After the MLCCC fractionation, Mv3glc represented 7% of the peak area and the catechin–Mv3glc adduct accounted for 53% of the peak area. HPLC–MS analysis of fraction 4 showed that the main peak corresponded to catechin–Mv3glc (*m/z* 781) and the secondary peak to catechin–catechin–Mv3glc (*m/z* 1069). This adduct results from nucleophilic addition of catechin–Mv3glc on the intermediate flavanol carbocation arising from taxifolin.

Gradual increase of the ratio of the absorbance value at 280 nm to the absorbance value at 520 nm was observed from Mv3glc to catechin–Mv3glc dimer and to catechin–catechin–Mv3glc trimer (Figure 10), as expected from their respective structures. As mentioned previously, further increase of this ratio was observed for the major peak eluted in the first CCC fraction (Figure 3), suggesting a larger proportion of catechin units (or colorless anthocyanin forms) in its structure, in agreement with the proposed (cat)$_n$–Mv3glc ($n > 2$) structure.

The structure of the dimeric F-A$^+$ adduct separated by CCC was confirmed by NMR and its color properties were studied (26)

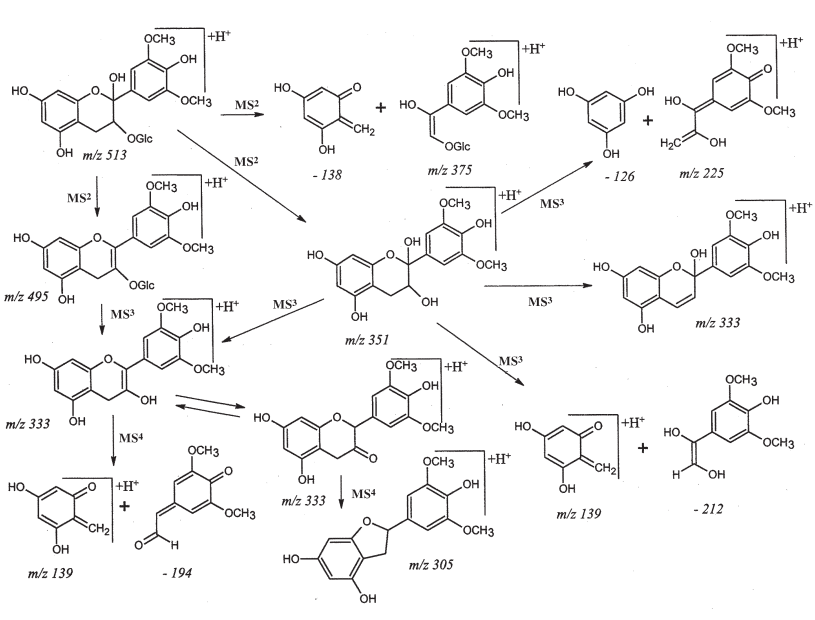

Figure 8. MS^2, MS^3, and MS^4 analysis of the molecular ion at *m/z* 513.

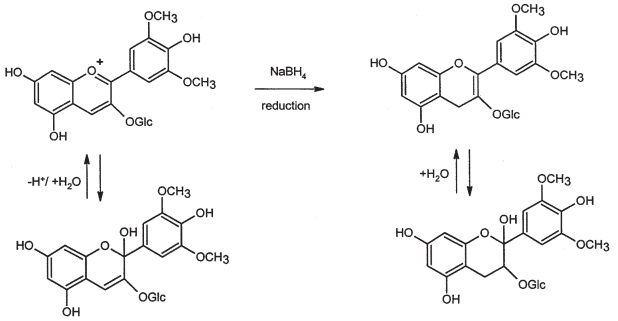

Figure 9. Possible formation pathway for molecular ions at *m/z* 511 and 513.

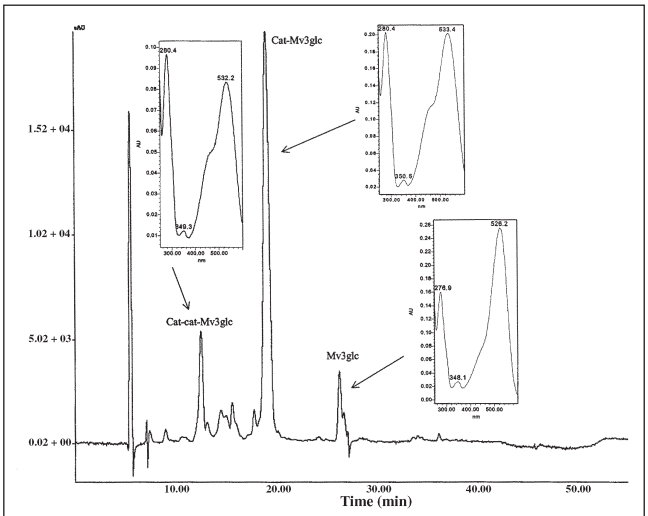

Figure 10. HPLC profile at 520 nm of fraction 4.

Conclusion

CCC has already proven to be an efficient tool for large-scale isolation of anthocyanins (6). It is also an excellent way of pre-fractionation, instead of classical gel chromatographic techniques or preparative HPLC, which are time-consuming. CCC allowed the separation of F-A$^+$ pigments obtained by synthesis from their anthocyanin precursor and the recovery of, on one hand, catechin–Mv3glc along with some $(catechin)_2$–Mv3glc and, on the other hand, polar polymeric pigments in fractions almost devoid of Mv3glc. Efficient fractionation and purification is one of the main prerequisites for obtaining sufficient quantities of newly formed pigments in order to study their structure and color properties.

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