Food Chemistry 121 (2010) 1129–1138

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

The fate of flavanol–anthocyanin adducts in wines: Study of their putative reaction patterns in the presence of acetaldehyde

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ARTICLE INFO

Article history:
Received 19 October 2009
Received in revised form 19 November 2009
Accepted 20 January 2010

Keywords:
Flavanol–anthocyanin adducts
Wine pigments
Hemisynthesis
Acetaldehyde
HPLC–ESI-MS

ABSTRACT

Methylmethine bridged flavanol–anthocyanin dimeric adducts (F–A) were synthesised by hemisynthesis using catechin, acetaldehyde and F–A adducts as precursors in order to simulate putative reactions undergoing in red wine. The products obtained, catechin–methylmethine–catechin–malvidin-3-*O*-glucose and methylmethine-(catechin–malvidin-3-*O*-glucose)$_2$, were analysed by HPLC–ESI-MS. The fragmentation patterns allowed the confirmation of the structures. These compounds were investigated and found in wine samples (2-year-old table wine and 10-year-old Port wine), thus confirming their formation *in vino* as a result of flavanol–anthocyanin adducts transformations.

1. Introduction

Anthocyanins are phenolic compounds belonging to the group of flavonoids and are responsible for plant pigmentation. They are often found in the epidermic tissue of flowers and fruits (Alcalde-Eon, Escribano-Bailón, Santos-Buelga, & Rivas-Gonzalo, 2006; Borkowski, Szymusiak, Gliszczynska-Swiglo, & Tyrakowska, 2005; Es-Safi et al., 2008; Vidal, Cartalade, Souquet, Fulcrand, & Cheynier, 2002). They occur as water soluble glycosides or acylglycosides, the latter being aliphatic, phenolic or hydroxycinnamic acid residues esterified to the sugar moieties (Borkowski et al., 2005). During the winemaking process, anthocyanins are extracted from grape berries, thus becoming directly responsible for the red wine colour (Alcalde-Eon et al., 2006; Borkowski et al., 2005). Other anthocyanin features such as antioxidant capacity, radical scavenging and metal chelation ability may also play an important role in wine quality (Vidal et al., 2002).

Monomeric anthocyanins do not remain stable for a long time in wine (Brouillard, Chassaing, & Fougerousse, 2003). In fact, it is believed that they undergo several types of reactions: (a) oxidation (Es-Safi et al., 2008); (b) aldehyde mediated condensation between anthocyanins (Abe et al., 2008; Drinkine, Lopes, Kennedy, Teissedre, & Saucier, 2007a; Gonzalez-Paramas et al., 2006); (c) direct condensation between anthocyanins (Alcalde-Eon, Escribano-Bailon, Santos-Buelga, & Rivas-Gonzalo, 2007; Lopes-da-Silva, Escribano-Bailon, & Santos-Buelga, 2007; Vidal, Meudec, Cheynier, Skouroumounis, & Hayasaka, 2004); (d) formation of a pyranic ring as a result of the reaction between anthocyanins and molecules bearing a vinyl moiety, such as acetaldehyde (B-type vitisins) (Alcalde-Eon, Escribano-Bailón, Santos-Buelga, & Rivas-Gonzalo, 2004; Drinkine et al., 2007a), pyruvic acid (A-type vitisins), acetoacetic acid (He, Santos-Buelga, Silva, Mateus, & de Freitas, 2006), vinylphenols or vinylflavonols (Alcalde-Eon et al., 2004); (e) aldehyde mediated condensation between anthocyanin and flavan-3-ols (Alcalde-Eon et al., 2004, 2006; Drinkine et al., 2007a; Gonzalez-Paramas et al., 2006); (f) direct condensation between anthocyanin and flavan-3-ols (Duenas, Fulcrand, & Cheynier, 2005; Remy, Fulcrand, Labarbe, Cheynier, & Moutounet, 2000; Salas et al., 2004a).

The direct condensation between flavan-3-ols and anthocyanins may originate both anthocyanin–flavanol (A–F) and flavanol–anthocyanin (F–A) adducts, depending on the reaction mechanism. These compounds were initially postulated by Jurd and Sommers in the late 1960s (Alcalde-Eon et al., 2004; Gonzalez-Manzano et al., 2008) but they remained a theoretical hypothesis until the late 1990s/early 2000s when their presence in red wines and chemical structures were demonstrated by HPLC–MS (Vivar-Quintana, Santos-Buelga, Francia-Aricha, & Rivas-Gonzalo, 1999), thiolysis (Remy et al., 2000), hemisynthesis (Salas et al., 2004a), mass fragmentation patterns (Gonzalez-Paramas et al., 2006; Salas et al., 2004a) and NMR (Gonzalez-Manzano et al., 2008; Salas, Le Guerneve, Fulcrand, Poncet-Legrand, & Cheynier, 2004b). In the last decade, this type of compounds was also detected and identified in plants such as strawberries (Fossen, Rayyan, & Andersen, 2004), beans, grapes (Gonzalez-

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doi:10.1016/j.foodchem.2010.01.060

Paramas et al., 2006) and corn (Gonzalez-Manzano et al., 2008; Gonzalez-Paramas et al., 2006).

The previous works demonstrated that the F–A adducts possess, when compared to their anthocyanic precursors, different colour properties (batochromic shift of 10 nm) but similar chemical stability towards hydration and sulphite bleaching (Salas et al., 2004b).

Even though the *in vitro* hemisynthesis of Cat–Mv3glc adducts became possible in 2004, no studies regarding their stability/reactivity in wine-like solutions were published since then.

The F–A adducts are composed by one flavan-3-ol moiety (upper unit) linked to an anthocyanin moiety (lower unit) by a B-type C_4–C_8 interflavanic bond. For that reason they should present typical reactivity of both flavanols and anthocyanins. So, they are expected to react, as nucleophiles at the C_6 and C_8 carbons of the flavanol upper unit and as electrophiles at the C_2 and C_4 carbons of the anthocyanin lower unit. As the latter kind of reactions were already regarded (Salas et al., 2004), the present work tried to focus on the possible reactions occurring in the upper unit of the adduct. Since red wines tend to have a high content in acetaldehyde either by yeast production or ethanol oxidation, the most probable reactions would be the formation of methylmethine bridged adducts (Drinkine, Lopes, Kennedy, Teissedre, & Saucier, 2007b). These methylmethine bridged compounds would be anthocyanin–methylmethine–adduct, flavan-3-ol-methylmethine–adduct and adduct–methylmethine–adduct.

In order to study new putative wine pigments such as these, a possible strategy is to chemically synthesize them starting from selected organic precursors. Structure elucidation may be subsequently conducted by mass and NMR spectroscopies (Brouillard et al., 2003).

Taking this strategy into account, both catechin–methylmethine–catechin–Mv3glc and catechin–Mv3glc–methylmethine–catechin–Mv3glc were synthesised, characterised (UV/vis and mass spectroscopy) and investigated in real wine samples. This work is the first report of the UV–vis spectra and mass fragmentation patterns of the products of the reaction between flavan-3-ol-anthocyanin dimeric adducts, flavan-3-ols and acetaldehyde.

2. Materials and methods

2.1. Reagents

Organic solvents, ethyl ether (pure) and ethyl acetate (pure) and methanol (pure) were purchased from José M. Vaz Pereira (Lisboa, Portugal) and acetonitrile (HPLC grade) and ethanol (Absolute) were purchased from Carlo Erba (Val de Reuil, France).

(+)-Catechin hydrate (*p*. 98%) and (−)-epicatechin (*p*. 98%) were purchased from Sigma–Aldrich (Madrid, Spain). (+)-Taxifolin (*p*. 98%) was purchased from Extrasynthèse (Genay, France).

Malvidin-3-*O*-glucoside (Mv3glc) was extracted and purified from a young red wine (*c.v.* Touriga Nacional) by semi-preparative HPLC with C_{18} reversed phase column, as described elsewhere (Pissarra, Mateus, Rivas-Gonzalo, Buelga, & de Freitas, 2003).

Sodium borohydride (*p*. 98%) was purchased from Sigma–Aldrich (Madrid, Spain). Acetaldehyde (*p*. 99%) and formic acid MS grade were obtained from Fluka (Buchs, Switzerland). Formic acid (*p*. 98%) and hydrocloric acid (37% v/v) were purchased from Panreac (Barcelona, Spain).

Procyanidin B4 dimer was hemisynthesised according to the procedure described by Pissarra et al. (2004).

2.2. Hemisynthesis of the adduct catechin-(4,8)–malvidin-3-O-glucoside (Cat–Mv3glc)

The hemisynthesis of the adducts Cat–Mv3glc was partially based on the works of Haslam (1980) and Hemingway and McGraw (1983) and consisted in a mild acidic cleavage of procyanidin in the presence of Mv3glc. In short, in a 50 ml schlenk under argon atmosphere, 60 mg of Mv3glc were placed in 15 ml of 10% formic acid aqueous solution, followed by 4 mg of procyanidin B4 and 30 ml of ethyl ether. After sealing, the schlenk was heated and stirred for 3 h at 85 °C. At the end, the organic phase was discarded. The addition of procyanidin and ethyl ether was repeated four times. The aqueous phase was then fractionated as described in Section 2.3.

2.3. Purification of the adduct Cat–Mv3glc

The fractionation and purification of the synthesised adduct was performed in three different stages:

2.3.1. Stage 1 – Rough fractionation

The evaporated aqueous phase of the procedure described in Section 2.2. was directly applied to 50 g of Toyopearl HW40(s) gel in a 10 cm diameter medium porosity sintered glass funnel with connected to standard vacuum filtration glassware and gradually eluted with increasing percentage (v/v) of acidified methanol (20%, 80% and 100%).

2.3.2. Stage 2 – Refined fractionation

The 80% (v/v) acidic methanolic solution was further fractionated by column (i.d. 16 mm) chromatography. The stationary phase was composed by 30 g of Toyopearl HW40(s) and the mobile phase by a series of 40% (v/v), 75% (v/v) and 100% (v/v) of acidic methanol. The flow rate of the peristaltic pump was set at 0.8 ml min^{-1}. The collection of the fractions was carried out by the visual detection of the coloured bands formed.

2.3.3. Stage 3 – Purification by preparative reversed phase HPLC

The specifications are described in Section 2.6.3. The eluted peaks were collected, frieze dried and kept away from the light at −18 °C until further use.

2.4. Hemisynthesis of Cat–Mv3glc derived compounds

2.4.1. Hemisynthesis of catechin–methylmethine–catechin-(4,8)–malvidin-3-O-glucoside

Cat–Mv3glc (0.32 μmol) and 500 μl of 20% aqueous ethanolic solution were transferred to a 750 μl microtube. The pH was adjusted to 3.0 with diluted hydroalcoholic HCl. It was then added 1.0 and 10.0 M equivalents of (+)-catechin and acetaldehyde, respectively. The microtube was sealed and incubated at 35 °C for 10 days. The reaction was followed both by HPLC–DAD and HPLC–MS.

2.4.2. Hemisynthesis of methylmethine-(catechin-(4,8)–malvidin-3-O-glucoside)$_2$

The procedure used for this synthesis was the same as the method described in the Section 2.4.1. without (+)-catechin.

2.5. Wine sample fractionation

Two different kinds of red wines were used to investigate the presence of the methylmethine bridged Cat–Mv3glc derivatives in wine: a 2-year-old red wine from the Douro Demarcated Region (Portugal) and a 10-year-old Vintage Port wine.

Both wine samples were processed similarly. Briefly, 750 ml of wine were dealcoholised in a rotary evaporator ($T \sim 35$ °C), applied to a reversed phase C_{18} placed into a 10 cm diameter medium porosity sintered glass funnel connected to standard vacuum filtration glassware, washed with water to remove the sugars, salts and other organic acids and finally eluted with acidic methanol to re-

move the phenolic compounds. The methanol was evaporated under vacuum in the presence of water (250 ml) and the remaining solution extracted with ethyl acetate (3 × 250 ml). The organic phases were discarded and the aqueous phases containing the red wine pigments were fractionated with 50 g of Toyopearl HW40(s) in a sintered glass funnel under vacuum and consecutively eluted with 200 ml of acidified water and 20% (v/v), 40% (v/v), 60% (v/v), 80% (v/v) and 100% (v/v) of aqueous acidic methanol. The fractions were concentrated, in a rotary evaporator, up to 10 ml of water and analysed by HPLC–MS prior to freeze-drying.

2.6. Chromatographic conditions

2.6.1. HPLC–DAD

The HPLC used was an ELITE LaChrom fitted with a L-2130 quaternary pump, a L-2200 autosampler and a L-2455 DAD. The stationary phase was a MERK RP – C_{18} column 250 mm × 4 mm i.d. (5 μm). The injection volume was 20 μl and the mobile phase was composed by two solvents: solvent A, a 10% (v/v) aqueous formic acid solution, solvent B, formic acid:acetonitrile:water (1:3:6) (v/v). The elution conditions were: flow rate set at 1 ml min^{-1}, starting with a linear gradient from 20% to 66% of B in 50 min, a second linear gradient from 66% to 100% of B for 10 min followed by an isocratic elution of 100% of B for another 10 min. The column was washed with 100% of acetonitrile for a 7 min period proceeded by a re-equilibration step of 15 min of isocratic 20% of B.

The UV–vis absorbance spectra were recorded from 250 to 700 nm and the peaks recorded at 280 and 520 nm.

2.6.2. HPLC–ESI-MS

The chromatographic mass spectra analyses were also performed in a Finnigan Surveyor Plus HPLC system fitted with a PDA Plus Detector, an Autosampler Plus and a LC quaternary pump plus. The HPLC system was coupled to a mass detector Finnigan LCQ Deca XP Plus equipped with an ESI source and an ion trap quadrupole.

The chromatographic conditions used were: a stationary phase composed by a Hypersil Gold 150 × 4.6 mm i.d. (5 μm) column (Thermofinnigan, CA). Injection volume was 25 μL. Mobile phase was composed by solvent A, 1% (v/v) of formic acid and solvent B, 1% (v/v) of formic acid and 30% (v/v) of acetonitrile in water. The flow rate was set at 0.5 ml min^{-1}. The chromatographic run started with a linear gradient from 20% to 85% of solvent B in 70 min followed by another linear gradient until 100% of solvent B for 5 min. At this point a cleaning step of 100% of solvent B for 10 min was performed and followed by 15 min re-equilibration time with the initial conditions.

The mass spectrometric conditions were: source and capillary voltages of 5 kV and 4 V, respectively. Capillary temperature of 325 °C. Nitrogen was used at flow rates of 90 and 25 as sheath and sweep gas, respectively. The mass spectra were recorded between 250 and 2000 *m/z*. A zoom scan of the most intense ion with 25 V collision energy for MS^2 and 30 V collision energy for MS^3 were also registered. For wine samples, LC–MS analyses were also performed in Selected Ion Monitoring mode (SIM) for the signals of *m/z* = 1097 and 794 followed by MS^2 and MS^3 fragmentations with the conditions described as above.

2.6.3. Semi-preparative HPLC

The semi-preparative HPLC system used was composed by a Knauer K-1001 quaternary pump, a rheodyne 7125 manual injector with a 200 μl loop and an UV SFD S3210 detector.

The stationary phase was a Thermofinnigan Hypersil Gold column 250 × 10 mm i.d. (5 μm).

The mobile phase was composed by solvent A, 10% (v/v) of formic acid, and solvent B, aqueous solution of 10% (v/v) formic acid and 30% (v/v) methanol. The rate flow used was 4 ml min^{-1}. The gradient method started with a linear gradient ranging from 20% B to 66% of B in 50 min followed by another linear gradient from 66% to 100% of B in 10 min. At that point, an isocratic step was maintained at 100% B for another 10 min proceeded by a re-equilibration step of 15 min of 20% of B.

3. Results and discussion

3.1. Hemisynthesis of Cat–Mv3glc adduct

The adducts Cat–Mv3glc were synthesised through a nucleophilic addition of a hemiketalic malvidin-3-glucoside to a catechin C_4 carbocation. The carbocation was obtained by a mild acidic cleavage of a dimeric procyanidin (B4). Salas et al. (2004a) have previously proposed a very similar hemisynthetic approach to synthesize this adduct (Salas, Fulcrand, Meudec, & Cheynier, 2003; Salas et al., 2004a), but the main issues were a very slow reaction kinetics and an apparent production in very small quantities. The problems were somewhat overcome herein with the application of heat in a biphasic solvent (H_2O/ethyl ether) system. The heating of proanthocyanidins in an acidic medium induced the production of a C_4 carbocation of catechin easily attacked by a nucleophile (*vide* Fig. 1), which in the present case is one of the carbons (C_6 or C_8) with negative charge density (δ^-) of the phloroglucinol ring A of malvidin-3-glucoside yielding the Cat–Mv3glc dimeric adduct.

The synthesis was carried out in a biphasic system in order to prevent the proanthocyanin regeneration resulting from the nucleophilic attack of the phloroglucinol ring of catechin to the carbocation that competes with Mv3glc (Vidal et al., 2002), and thus enhance the reaction yield which was 12% (checked by HPLC–DAD). The organic phase – ethyl ether – grasped the epicatechin molecules released by the procyanidin acid decomposition away from the aqueous phase, where the reaction between the carbocation and Mv3glc occurs.

The addition of small molar ratio quantities of procyanidins ensured that the concentration of Mv3glc is much higher than the catechin unit released, improving the synthesis of Cat–Mv3glc.

The purification steps were modified given the inexistence of conditions to perform preparative countercurrent chromatography. The purification process proposed herein also allows an easy scale-up.

To investigate the purity and unequivocally identify the synthesised adduct, an NMR analysis was carried out. The results were in agreement with the ones reported in the literature (Salas et al., 2004b).

3.2. Hemisynthesis of Cat–Mv3glc derived compounds

3.2.1. Catechin–methylmethine–catechin-(4,8)–malvidin-3-O-glucoside

It is well known that flavanols and anthocyanins, in the presence of acetaldehyde, condense with each other through methylmethine (Mm) bridges (Es-Safi, Cheynier, & Moutounet, 2002; Es-Safi, Fulcrand, Cheynier, & Moutounet, 1999; Escribano-Bailon, Alvarez-Garcia, Rivas-Gonzalo, Heredia, & Santos-Buelga, 2001; Saucier, Little, & Glories, 1997). Bearing this, it would be expected at least four positional isomers: Cat-8–Mm and Cat-6–Mm bridged radicals condensed into the C_8- and C_6-A ring (catechin moiety) of the Cat–Mv3glc adduct (Fig. 2). Furthermore, there is also the possibility of occurring diastereoisomers differing in the configuration (R/S) of the asymmetric carbon of the methylmethine bridge (Escribano-Bailon et al., 2001). Therefore, the number of compounds expected in this reaction was at least eight isomers.

The chromatogram of the reaction mixture is shown in Fig. 3. Several peaks with retention times ranging between 30 and 60 min showed mass/charge ratios (m/z = 1097) similar to the compound catechin–Mm–catechin-(4,8)–malvidin-3-*O*-glucoside.

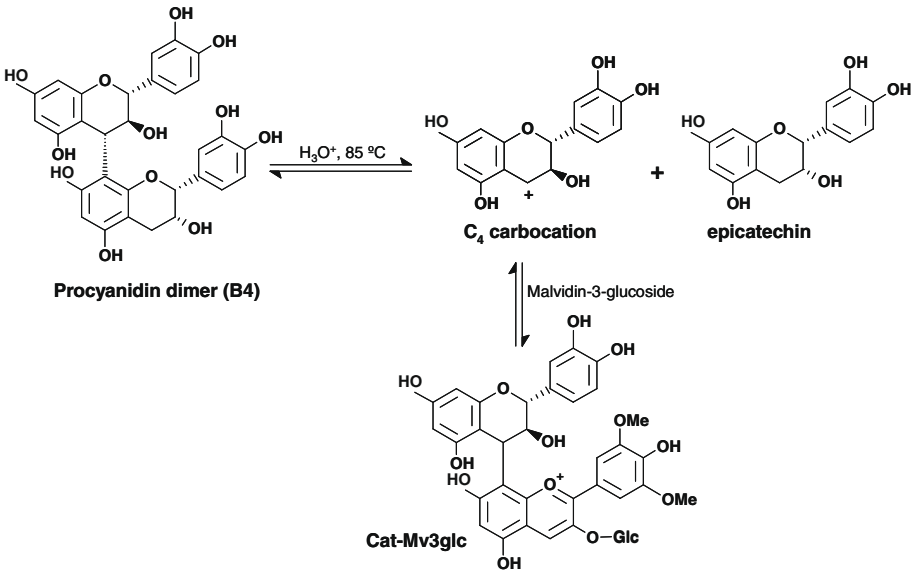

Fig. 1. Proposed reaction mechanism between procyanidin dimers and malvidin-3-glucoside under acidic conditions.

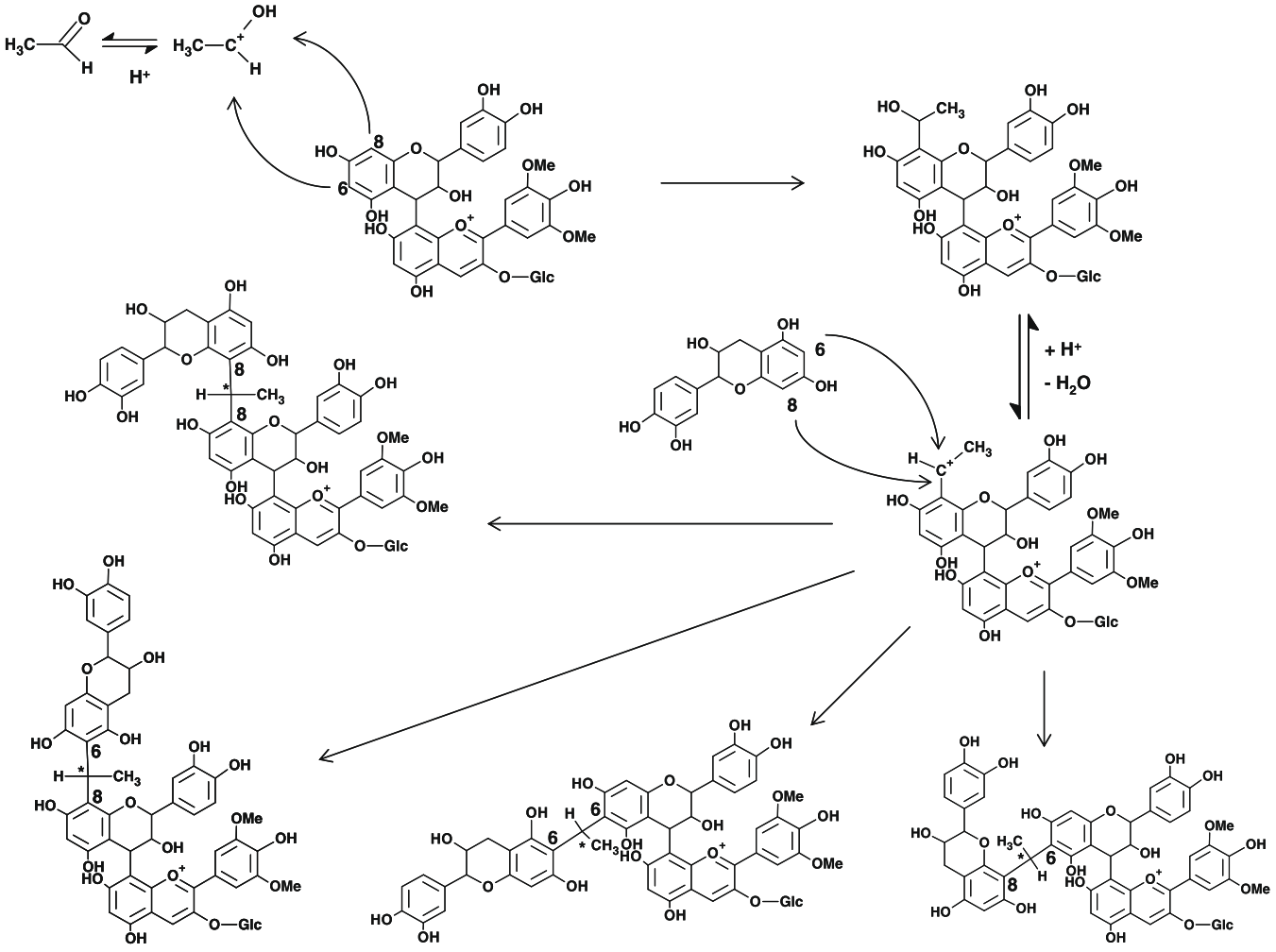

Fig. 2. Proposed reaction mechanism between catechin, acetaldehyde and Cat–Mv3glc. The structures of the four positional isomers are showed.

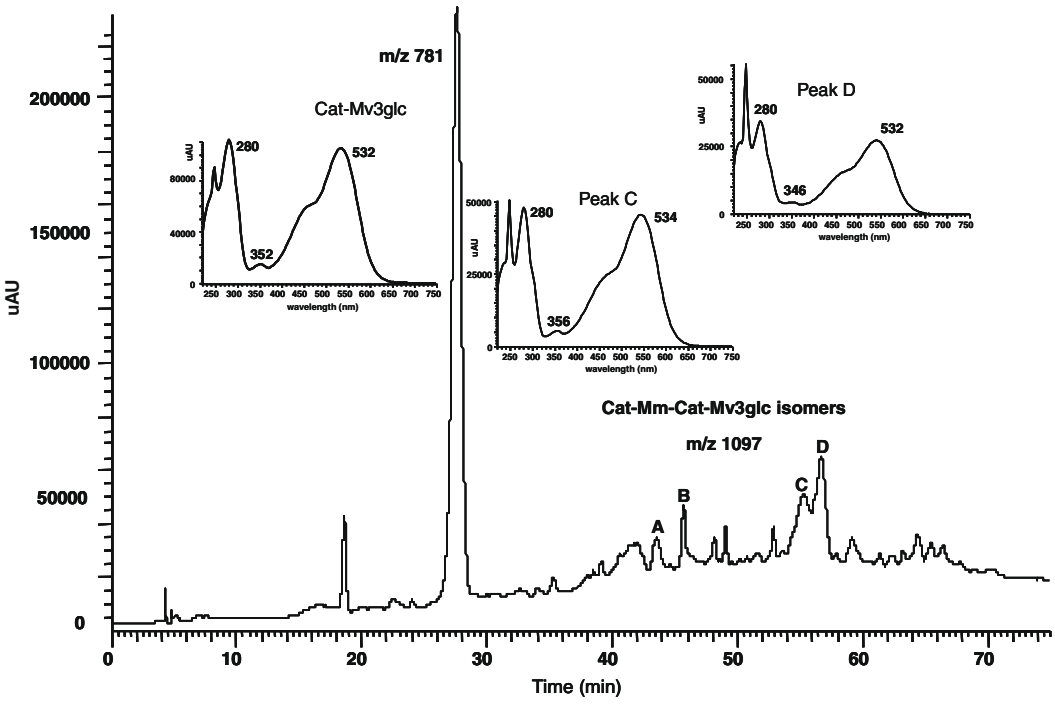

Fig. 3. Chromatogram (520 nm) of the reaction between Cat–Mv3glc and catechin in the presence of acetaldehyde. The main peaks with a mass/charge ratio of 1097 are noted with capital letters A–D. Inset: Cat–Mv3glc and peaks C and D UV–vis spectra. Every noted peak present similar spectra.

Only the four major peaks noted by A–D (Fig. 3) presented consistent fragmentations. Fig. 3 only shows the UV-vis spectra of two of the noted peaks, since they are similar.

The fragmentation patterns are consistent with the structure proposed (see Table 1). Peaks C and D are the most intense and may correspond to the two diastereoisomers R and S resulting from the condensation involving the C_8 carbon of the phloroglucinol ring of both catechin moieties through the methylmethine bridges by similarity with the catechin–methylmethine dimers described in the literature (Drinkine, Glories, & Saucier, 2005).

The two most common signals in the fragmentation pattern of the molecular ion were $m/z = 935$ and 807 which correspond to the loss of the glucose moiety ($[M-162]^+$) and the loss of both the catechin unit with subsequent rearrangement of the ethyl to ethylene ($[M-289-1]^+$), respectively (Fig. 4). The rearrangement of the ethyl substituent was previously described by Saucier et al. (1997).

The signal at $m/z = 783$ arises from the loss of a glucose moiety followed by the loss of the B ring, by retro Diels–Alder (RDA) decomposition in the catechin moiety of the adduct ($[M-162-152]^+$).

The signal at $m/z = 645$ arise from the loss of catechin unit and subsequent rearrangement of the ethyl substituent to ethylene followed by the loss of glucose ($[M-162-289-1]^+$) or *vice versa* ($[M-289-1-162]^+$).

The mass fragment at $m/z = 619$, already described (Salas et al., 2004a), resulted from the loss of catechin–methylmethine substituent and glucose moiety.

The signal $m/z = 493$, also previously described (Salas et al., 2004a), arises from the loss of the A ring, by RDA decomposition from both the aglycone fragments of Cat-(4,8)–Mv ($[M-317-162-126]^+$) and catechin–Mm–catechin-(4,8)–malvidin ($[M-162-442]^+$). Peak B displayed some unidentified signals ($m/z = 1042$, 875 and 693).

As seen in Table 1, all compounds presented wavelength maxima at 280 and 532 nm, a shoulder in the visible band at 472 nm coherent with the glycosilation at carbon C_3 (Santos-Buelga, García-Viguera, & Tomás-Barberán, 2003) and a small band at 346 nm. Altogether, the values are consistent with the presence of a Cat–Mv3glc moiety in the molecule (Salas et al., 2004a).

Table 1
Spectral characteristics and fragmentation patterns of the peaks A–H from the chromatograms (Figs. 4 and 7) of the reaction between catechin, acetaldehyde and Cat–Mv3glc adduct. The most intense peaks in MS^2, MS^3 and the UV–vis maxima are underlined.

Peak	UV–vis λ_{max}	m/z	m/z in MS^2	m/z in MS^3
A	280, 346, 472, 532	1097	935, 783, 645, 619, 493	783, 645, 619, 493
B	280, 346, 472, 532	1097	1042, 875, 807, 693, 645	–
C	280, 356, 534	1097	935, 807, 619, 535, 493	–
D	280, 346, 472, 532	1097	934, 806	–
E	280, 352, 468, 532	794	713, 632	645, 637, 632, 619
F	283, 472, 533	794	713, 623	645, 632, 618
G	283,469, 532	794	–	–
H	280, 468, 533	794	713, 632	645, 632, 619, 556

3.2.2. Methylmethine-(catechin-(4,8)–malvidin-3-O-glucoside)$_2$

This double charged pigment was synthesised by reaction between the Cat–Mv3glc adducts and acetaldehyde. In this reaction, was also expected the formation of some positional and diastereomeric isomers.

Four peaks with signal at $m/z = 794$ noted with E–H and other minor peaks (not shown) were identified in the chromatogram of the reaction (Fig. 5).

These peaks showed both similar UV–vis and mass spectra (Table 1 and Fig. 5). There were a few negligible shifts in the wave-

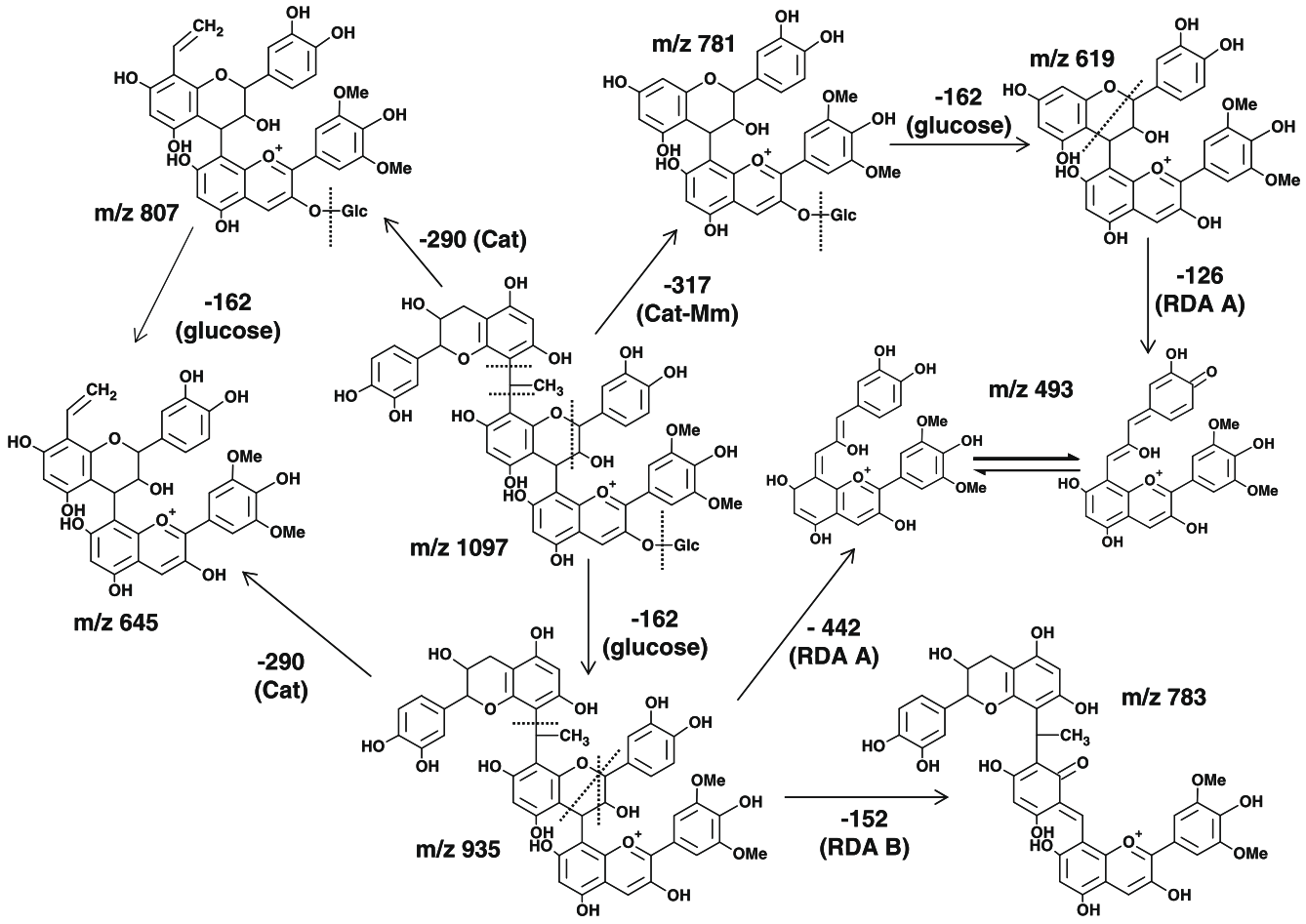

Fig. 4. Fragmentation pattern of the molecule Cat-(8,8)–methylmethine–Cat-(4,8)–Mv-3-Glc in the positive ion mode.

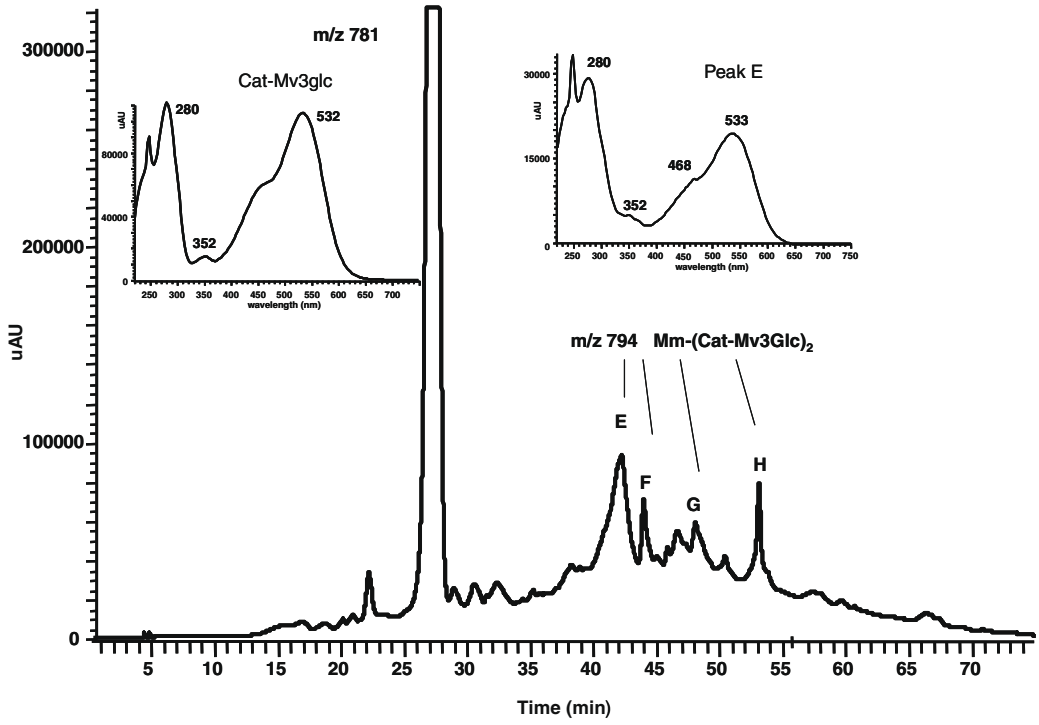

Fig. 5. Chromatogram (520 nm) of the reaction between Cat–Mv3glc and acetaldehyde. The peaks of interest with a mass/charge ratio of 794 are noted with capital letters E–H. Inset: Cat–Mv3glc and peak E UV–vis spectra. Every noted peak present similar spectra.

length maxima λ_{max} but the overall spectra were similar to the spectrum of Cat–Mv3glc.

The UV–vis spectral similarity to Cat–Mv3glc was already anticipated because the molecule is composed by two Cat–Mv3glc molecules bonded through a methylmethine bridge.

Although the molecule possesses a mass of 1588 Da, it originated a mass to charge signal ratio of 794 since it presented two charges (at each malvidin moiety). This was confirmed by a zoom scan which showed isotopic *m/z* signals with a 0.5 Da difference (Fig. 6).

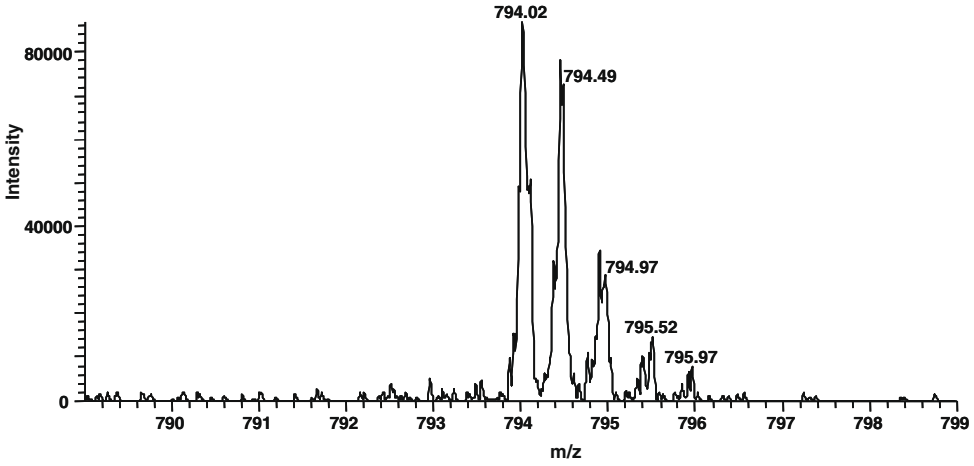

Fig. 6. Zoom scan of the peak E, with the *m/z* ratio of 794.

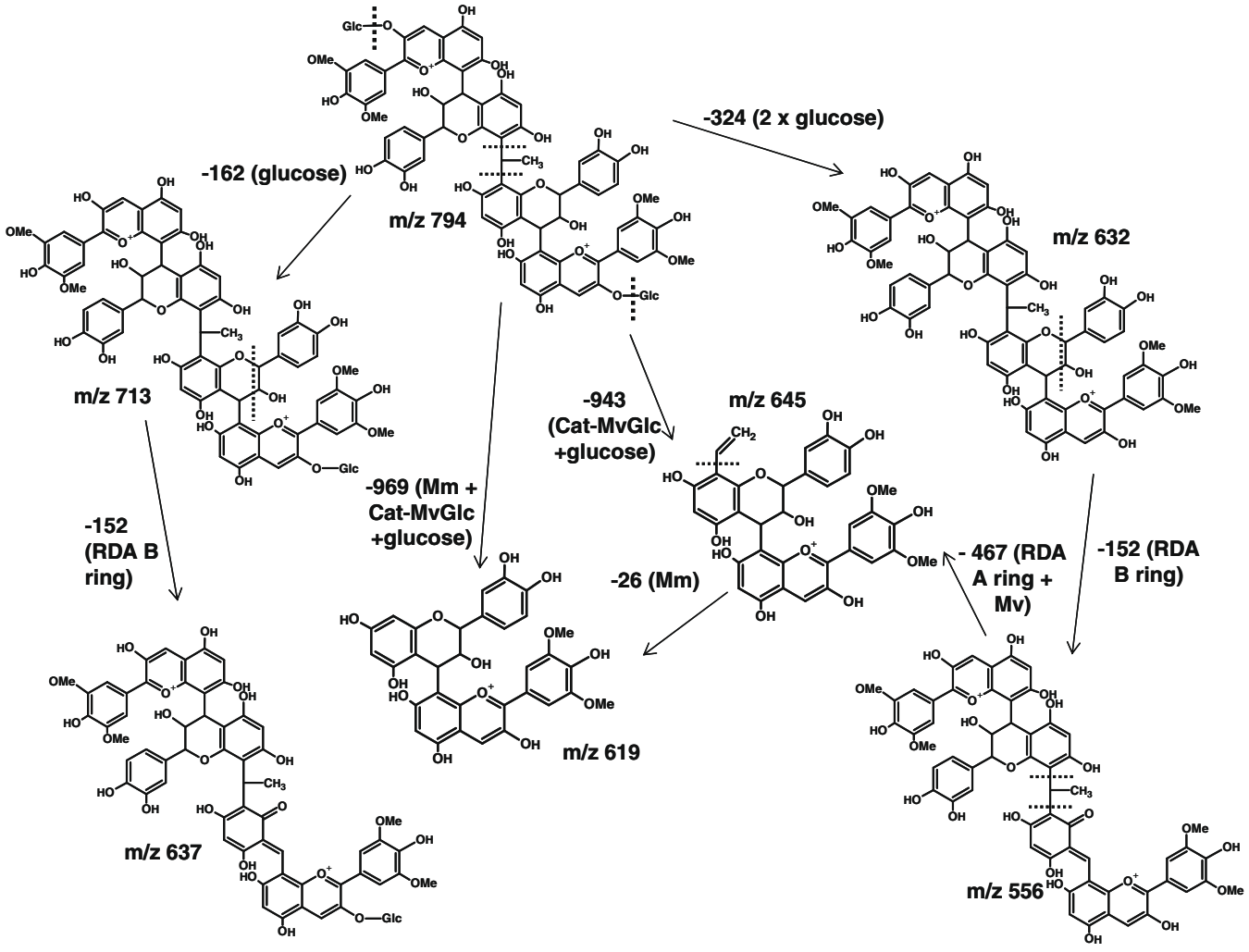

Fig. 7. Main fragmentation pattern of the molecule Mm–(Cat-(4,8)–Mv3Glc)2 in positive mode.

The loss of one or two glucose moieties originated signals at *m/z* = 713 and 632, respectively. The loss of glucose moieties as the most common fragmentation is consistent with the fragmentation pattern of Cat–Mv3glc (Salas et al., 2003, 2004a).

The fragmentation of the *m/z* = 794 ion in peaks E and H also yielded the signals at *m/z* = 637 and 556. Those signals may be interpreted as the loss of one glucose moiety and one B ring in a catechin unit as a result of a RDA fission and the loss of two glucose moieties and one B ring through a RDA fission, respectively (Fig. 7).

In the MS^3 spectra, the most intense ion at *m/z* = 713 originated mostly the fragments with *m/z* = 632 (loss of the other glucose moiety), 645 (loss of Cat–Mv) and 619 (loss of Mm–Cat–Mv), the last being the most common fragment of the Cat–Mv3glc adduct under the same fragmentation conditions (Salas et al., 2004a).

These compounds were also detected in the reaction between catechin, Cat–Mv3glc adducts and acetaldehyde, showing a competition between flavan-3-ols and Cat–Mv3glc adducts as nucleophiles in the addition reaction.

The yield of both reactions to obtain individual Cat–Mm–Cat–Mv3glc and Mm–$(Cat–Mv3glc)_2$ compounds were shown to be poor. In fact, the chromatograms of Figs. 3 and 5 are very complex showing many chromatographic peaks and humps corresponding to unidentified products resulting from side reactions involving the reagents (acetaldehyde, Cat–Mv and catechin) that are very likely to polymerize. On the other hand, those reactions lead to the formation of a relatively high number of isomeric products; four isomers were tentatively identified by mass spectrometry for each reaction (eight isomers were expected based on the mechanism described). Those factors and the reagents involved contribute together to the unsuccessful attempts performed to isolate pure individual compounds in enough amounts for their characterisation by NMR.

3.3. Presence of the Cat–Mv3glc derivatives in wine

The compounds previously synthesised were searched in two different kinds of red wine samples: a table red wine (2-year-old) and a Port wine (10-year-old) (Table 2). The compound Mm–$(Cat–Mv3glc)_2$ was only found in Port wine, whereas Cat–Mm–Cat–Mv3glc was only found in the 2-year-old wine sample.

3.3.1. Two-year-old red wine sample

The six aqueous wine fractions were subjected to HPLC–MS analysis. The fraction of 20% (v/v) acidified aqueous methanol which revealed the presence of peaks with a signal of 1097 Da (putative correspondence to Cat–Mm–Cat–Mv3glc) was also analysed by LC–MS SIM mode for that same signal, showing eight peaks (Fig. 8). The chromatographic retention times of these peaks are close to the ones from the synthesised Cat–Mm–Cat–Mv. It is possible that some of them are derivatives of epicatehin which have the same molecular weight, and the mass fragmentation is expected to be similar.

Peaks 1–5 presented a fragmentation pattern consistent with the one displayed by Cat–Mm–Cat–Mv3glc previously synthesised: a molecular ion with *m/z* = 1097, which originated the ionic fragments with *m/z* signals of 935 (main peak), 807, 781, 645 and

Table 2
Fragmentation patterns of the peaks 1–10 from the 2-year-old and 10-year-old wine samples analysed by HPLC–DAD–MS in SIM mode. The most intense peaks in SIM and MS^2 are underlined.

Wine sample	Peak	*m/z*	*m/z* in MS^2	*m/z* in MS^3
2-Year-old	1	1097, 1098	935, 781, 631, 619, 493	781, 631, 619, 494
	2	1097, 1098	1015, 935, 807, 783, 744, 765, 646, 631, 619, 493	781, 643, 631, 619
	3	1097, 1098	935, 807, 619, 535, 493	781, 631, 619, 494
	4	1097	935, 807, 781, 730, 630, 493	876, 781, 644, 631, 619, 494
	5	1097	1016, 935, 852, 807, 783, 659, 619, 602	645, 637, 632, 619
	6	1097	935, 875, 765, 519, 493	645, 519, 357
	7	1097	935, 917, 773, 631, 519, 357	887, 729, 644, 357
	8	1097, 1098	935, 519, 357	519, 357
10-Year-old	9	794	875, 775, 713, 698, 644, 632, 548, 374	356, 222
	10	794	746, 714, 546, 631, 596, 555, 492, 438	433, 410

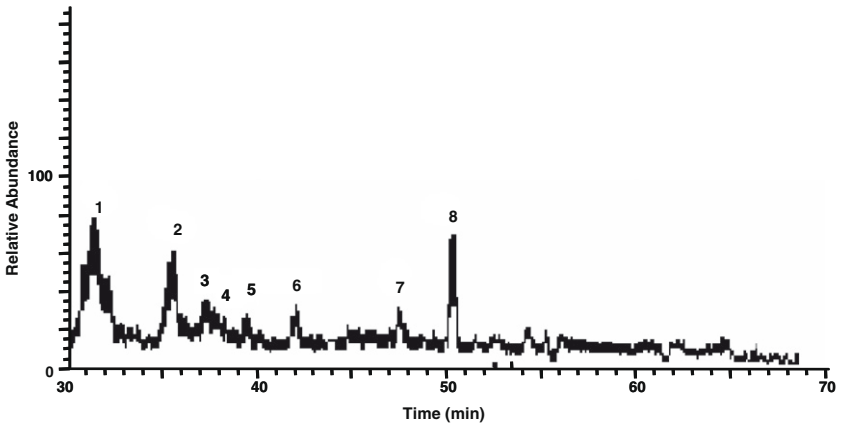

Fig. 8. Mass chromatogram (SIM at *m/z* = 1097) of the 20% methanolic fraction from a 2-year-old Douro wine. The peaks with signal 1097 are noted with numbers from 1 to 8.

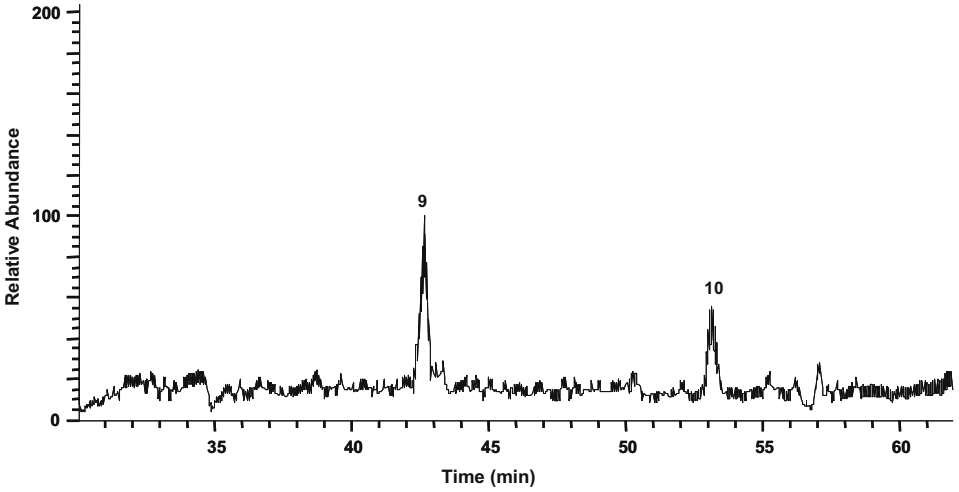

Fig. 9. Mass chromatogram (SIM at $m/z = 794$) of the 20% methanolic fraction from a Port wine. The peaks with signal 794 are noted with the numbers 9 and 10.

619. The most intense signal of MS^2 fragmentation, at $m/z = 935$ also refragmented into 807, 781, 645 and 619.

Peaks 6–8 presented a molecular ion with a mass/charge signal of 1097 and a main fragmentation signal at $m/z = 935$ due to the loss of a glucose moiety, but lacked the presence of the signal at $m/z = 807$, 781, 645 and 619. Instead, the fragments at $m/z = 357$ and 519 emerged as second and third more intense peaks of the fragmentation of the molecular ion. The signal at $m/z = 357$ was also a fragment of 935. The signal at $m/z = 357$ (330 + 27) and 519 (492 + 27) may correspond to malvidin and malvidin-3-glucoside linked to a vinyl group as a result of a loss of the catechin unit in a Cat–Mm moiety. The signal at $m/z = 519$ could also be a vinyl moiety linked to the malvidin unit of the RDA fragment (loss of the A ring) of the Cat–Mv3glc adduct. These fragments are not compatible with the ones yielded from the molecule Cat–Mm–Cat–Mv3glc even though they would be possible in the molecule Cat–Cat–Mm–Mv3glc, the latter one being already proposed by Atanasova, Fulcrand, Cheynier, and Moutounet (2002) and Alcalde-Eon et al. (2004) resulting from the classical condensation of procyanidin dimer and anthocyanins mediated by acetaldehyde resulting from oxidative wine ageing.

3.3.2. Port wine sample

The fractions obtained from Port wine were analysed by the same procedure as the 2-year-old red wine.

Only the 20% (v/v) methanolic fraction showed peaks with signal at $m/z = 794$. That fraction was reanalysed by HPLC–MS in the SIM mode set at 794. Two peaks (retention times of 42.7 and 53.2 min) were detected with that mass/charge value (Fig. 9). Both peaks fragmented generating signals at $m/z = 713$ and 632 which are consistent with the structure of Mm–(Cat–Mv3glc)$_2$ (Table 1).

The retention time and mass data are consistent with the structure of peaks E and H in the chromatogram of Fig. 5.

4. Conclusions

The catechin-(4,8)–malvidin-3-*O*-glucoside dimeric adducts, bearing a flavan-3-ol upper unit can undergo some of the characteristic reactions of the flavan-3-ols in wine forming methylmethine bridged compounds. This was demonstrated either by their hemisynthesis, in hydroalcoholic solutions in the presence of acetaldehyde and catechin, and by detection in red wine samples.

For the first time it was possible to present the UV-vis spectra and mass fragmentation patterns of the Cat–Mm–(Cat–Mv3Glc) and Mm–(Cat–Mv3Glc)$_2$ and devise a HPLC–MS method for their detection in wine, although this method is eminently qualitative.

These results are important because they bring insights of a part of the chemical composition of red wines that is still practically unknown.

Acknowledgements

The authors thank the financial support of FCT (Fundação para a Ciência e Tecnologia). F. Nave is recipient of a PhD Scholarship SFRH/BD/30294/2006. The authors also thank Dra. Zélia Azevedo for the LC–MS analysis.

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