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Analytical Methods

Study of hydroxycinnamic acids and malvidin 3-monoglucoside derivatives using capillary zone electrophoresis and ultra-performance liquid chromatography

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ABSTRACT

Model solutions (pH = 3.5, 12% ethanol) of malvidin 3-*O*-glucoside (Mv3glc), the most common free anthocyanin in grapes and red wines from *Vitis vinifera*, and three free hydroxycinnamic acids present in wines (caffeic, ferulic and *p*-coumaric acids) were studied.

The stability of the precursors and their derivatives was studied during a storage period of six months at 20 °C. Capillary zone electrophoresis with UV–vis detection (CZE/UV–vis) was used to monitor changes in sample composition. Ultra-performance liquid chromatography coupled to mass detection (UPLC/MS) was used to confirm the presence of the compounds formed. Discussion on the migration times of the new pigments as well as on their UV–vis and mass spectra is reported.

Six derivatives were detected in model wine solutions containing Mv3glc and caffeic acid. The six derivatives were identified as three condensation products of caffeic acid, 4-vinylcatechol, 4-vinylsyringol and a compound with a molecular ion at *m/z* 803, whose structure has been tentatively assigned to a caffeic acid linked to Pinotin A. The derivatives 4-vinylphenol and 4-vinylguaiacol pyranoanthocyanins were detected in solutions containing malvidin 3-*O*-glucoside together with *p*-coumaric and ferulic acid, respectively. The malvidin 3-*O*-glucoside derivative, 4-vinylsyringol was also detected in all solutions containing the anthocyanin regardless of the presence of hydroxycinnamic acid.

1. Introduction

Hydroxycinnamic acids are present as tartaric esters in vacuoles of grape skin and pulp cells of wine grapes and are named caftaric, *p*-coutaric and fertaric acid. Caftaric acid is by far the most predominant hydroxycinnamic acid in gapes and wine followed by *p*-coutaric acid and, albeit in smaller amounts, fertaric acid (Ong & Nagel, 1978). These naturally-occurring esters are susceptible to hydrolysis by the cinnamyl esterase enzyme (Somers, Verette, & Pocock, 1987), and this occurs in the aqueous acidic solution of wine, releasing the free hydroxycinnamic acids, which are readily detected in a few weeks old wine (Singleton, Zaya, & Trousdale, 1986).

The reaction between anthocyanins and hydroxycinnamic acids has gained importance, since it causes the stabilization of coloured forms of anthocyanins and consequently enhances their colour (Darias-Martín et al., 2002; Gris, Ferreira, Falcão, & Bordignon-Luiz, 2007; Mazzaracchio, Pifferi, Kindt, Munyaneza, & Barbiroli, 2004). The literature describes mechanisms involved in the formation of vinylphenolic pyranoanthocyanins, either by direct reaction of hydroxycinnamic acids and anthocyanins (Rentzsch, Schwarz, Winterhalter, & Hermosín-Gutierrez, 2007), or direct reaction of the anthocyanin with the 4-vinylphenol, emerging this last derivative from the decarboxylation of the corresponding hydroxycinnamic acid due to the enzymatic activity of *Saccharomyces cerevisiae* during fermentation (Schwarz, Wabnitz, & Winterhalter, 2003a; Morata, González, & Suárez-Lepe, 2007).

Direct reactions (pure chemical formations) of free hydroxycinnamic acids and anthocyanins have been described as responsible for the slow formation of pyranoanthocyanins during the maturation of red wines (Chatonnet, Dubourdieu, Boidron, & Lavigne, 1993; Rentzsch et al., 2007). These anthocyanin-vinylphenol-type adducts have been described as potentially attractive for use as ageing indicators for red wines as their concentration constantly increases during storage as long as free anthocyanins and cinnamic acids are available (Schwarz et al., 2003a).

Abbreviations: Mv3glc, malvidin 3-*O*-glucoside; CAFD, caffeic acid derivative; FERD, ferulic acid derivative; COUD, coumaric acid derivative; CZE, capillary zone electrophoresis; HPLC, high performance liquid chromatography; DAD, diode array detector; UV–vis, ultraviolet–visible; MS, mass spectrometry; UPLC, ultra-performance liquid chromatography.

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Several methods have been developed to analyse hydroxycinnamic acids by HPLC with diode array detection or coupled to mass spectrometry, proving to be the most powerful analytical separation and identification methods. These methods have been used to study the different hydroxycinnamic acids and their derivatives not only in wine samples (Darias-Martín et al., 2002; Oliveira, De Freitas, Silva, & Mateus, 2007) but also in cherries (Mozetic, Simcic, & Trebse, 2006) or maize bran (Rouau et al., 2003).

The absence of commercially available standards of many polyphenol derivatives such as flavanol, anthocyanin or hydroxycinnamic acid derivatives makes the study of the evolution of their precursors in model solutions essential and very helpful for their further identification in real wine samples. Many studies carried out by HPLC/UV–vis with hydroxycinnamic acids and Mv3glc have been performed in model solutions with a view to gaining knowledge on these compounds (Gris et al., 2007; Schwarz et al., 2003a; Gómez-Miguez, González-Manzano, Escribano-Bailón, Heredia, & Santos-Buelga, 2006; Oliveira et al., 2007).

Recently, CZE has attracted interest as a favourable technique for the determination of polyphenols in real wine samples (Guadalupe, Soldevilla, Sáenz-Navajas, & Ayestarán, 2006; Pazourek et al., 2005; Sáenz-López, Fernández-Zurbano, & Tena, 2004) as it offers several advantages such as the ability to separate condensed pigments, speed, small sample requirements or extremely limited solvent waste. Despite the advantages presented by CZE/UV–vis, only a few studies have provided information on the electrophoretic behaviour of polyphenol derivatives present in wines.

Consequently, the study in model solutions of free hydroxycinnamic acids and Mv3glc by CZE/UV–vis is of great interest due to the advantages offered by this technique in wine analysis and the high involvement of the derivatives of these phenolic compounds in wine colour and stability.

Therefore, the aim of this study was to examine the formation of derivatives of hydroxycinnamic acids and Mv3glc, as well as to evaluate their stability in model solutions by CZE/UV–vis. Different solutions of the main compounds involved in these reactions were prepared and regularly analysed by CZE/UV–vis and UPLC/MS. The evolution study of each compound was useful to establish the order of appearence/dissappearance, and to provide information about the relationships present between precursors and derivatives, thus enabling their stability to be evaluated.

2. Materials and methods

2.1. Reagents

Disodium tetraborate, HPLC-grade methanol, acetic acid and ethanol were supplied by Merck (Darmstadt, Germany), sodium hydroxide by Prolabo (France), tartaric, caffeic and *p*-coumaric acids by Sigma–Aldrich (Steinheim, Germany). Malvidin 3-*O*-glucoside chloride was purchased from Extrasynthese (Genay, France) and ferulic acid from Alfa Aesor (Heysham, United Kingdom). HPLC/MS-grade acetonitrile was provided by Scharlau Chemie (Spain).

Deionized water was purified with a Milli-Q water system (Millipore, Molsheim, France) prior to use.

2.2. Preparation of model solutions

Six solutions and a reference solution were prepared in a wine-like medium containing 5 g l^{-1} of tartaric acid in 12% ethanol and adjusted to a pH of 3.5 with 0.1 N NaOH. The solutions contained caffeic acid (solution 1), ferulic acid (solution 2), *p*-coumaric acid (solution 3), and the same hydroxycinnamic acids with Mv3glc using a molar ratio of 1:1 (solutions 4, 5 and 6, respectively). The reference solution contained the same anthocyanin concentration (0.41 M). Solutions were placed in vials and stored in darkness at 20 °C. Samples were taken periodically over 6 months. The first set of samples (day 0) was collected 30 min after preparation of solutions.

2.3. CZE/UV–vis analysis

Capillary zone electrophoresis was performed using an Agilent CE instrument (Waldbronn, Germany) equipped with a standard cassette containing an uncoated fused-silica capillary and a diode array detector.

The capillary was conditioned before injection by first washing with 0.1 N sodium hydroxide for 2 min, then with ultrapure water for 2 min, and finally with running buffer for 5 min. The buffer vials were automatically replenished after each run in order to use fresh buffer solution each time and improve the reproducibility of migration times.

The running buffer was 50 mM sodium tetraborate buffer solution (pH 9.4) containing 10% methanol (v/v) as modifier. A capillary with an effective length of 56 cm and an internal diameter of 75 μm, a voltage of 25 kV and an average current of 110 μA were used. The capillary temperature was maintained at 10 °C and the samples were injected in hydrodynamic mode at 50 mbar for 6 s (30 nl sample volume or 6 mm plug length). Electrophoregrams were recorded at 280, 520 and 599 nm and the spectrum from 200 to 599 nm was also collected for each peak. All the analyses were performed in triplicate and the results were expressed as mean values.

Peak assignations were based on the migration times and on their UV–vis spectra. Peak area, expressed as the percentage of the signal for the derivatives formed, was calculated related to the corresponding hydroxycinnamic acid precursor signal and in the case of the derivatives of Mv3glc and the acids related to the anthocyanin.

2.4. UPLC/ESI–TOF MS analysis

Ultra-performance liquid chromatography (UPLC) coupled to a mass detector was used to verify the chemical composition of solutions. This technique provided the sensitivity to tentatively identify the products formed in the solutions studied.

UPLC analyses were performed using a waters acquity ultra-performance LC system (Waters). UPLC separation was achieved using an acquity BEH C18 column (50 mm × 2.1 mm, i.d., 1.7 μm particle size, Waters), maintained at 25 °C, with a mobile phase flow rate of 0.2 ml min^{-1} and an injection volume of 1.0 μl. Solvents were (A) water/acetic acid (pH = 2.6), and (B) acetonitrile. The gradient programme employed was as follows: 0–2 min, 97–90% A; 2–6.8 min, 90–76%; 6.8–10.8 min, 76–60%; 10.8–11 min, 60–97%A; 11–15 min, 97%A.

The UPLC system was coupled to a micrOTOF mass spectrometer (Bruker Daltonik, Germany) equipped with an Apollo II ESI/APCI (electrospray/atmospheric pressure chemical ionisation) multimode source and controlled by Bruker Daltonics DataAnalysis software. The electrospray source was operated in positive mode.

The capillary potential was set to 2.5 kV; drying gas temperature was 200 °C and the flow 5 l min^{-1}; the nebulizer gas was set to 2 Bar and 200 °C. Spectra were acquired between *m/z* 90 and 3000.

Peak assignations were based on the retention times and on their MS spectra. Peak area, expressed as the percentage of the signal for the derivatives formed, was calculated related to the corresponding hydroxycinnamic acid precursor signal and in the case of the derivatives of Mv3glc and the acids related to the anthocyanin.

The evolution study of the peak areas carried out by both techniques, CZE/UV–vis and UPLC/MS, provided with tentative assignations of peaks.

3. Results and discussion

3.1. Study of caffeic acid solutions

Solution 1 was prepared in order to analyse the behaviour of caffeic acid and to study the formation of new derivatives of the acid in a model system. Three derivatives of caffeic acid were detected by CZE/UV–vis and the following codes were used for naming them, where CAFD denotes caffeic acid derivative: CAFD1, CAFD2 and CAFD3. Their migration times are listed in Table 1. Fig. 1a shows the evolution of the derivatives formed. It can be observed that they presented two distinguishable stages: the first with a faster formation period; and a second where compounds remained practically constant after 100 days of reaction. Derivatives with lower charge-to-size ratios (CAFD1 and CAFD2) formed within the first 20 days of reaction, whereas CAFD3 presented a higher charge-to-size ratio as it migrated later and was first detected after 70 days of reaction.

Table 1
Migration times (t_m) of the compounds separated by CZE.

compound	t_m, min (RSD,%)	compound	t_m, min (RSD,%)
CAFFEIC ACID	29.7 (0.03)	FERD1	24.80 (0.02)
CAFD1	20.90 (0.02)	FERD2	28.30 (0.01)
CAFD2	26.50 (0.03)	FERD3	14.50 (0.02)
CAFD3	27.30 (0.03)	*p*-COUMARIC ACID	36.3 (0.03)
CAFD4	14.50 (0.02)	COUD1	27.60 (0.03)
CAFD5	16.40 (0.04)	COUD2	29.30 (0.02)
CAFD6	18.20 (0.02)	COUD3	37.90 (0.03)
FERULIC ACID	29.9 (0.04)	COUD4	14.50 (0.02)

In order to identify the compounds detected by means of CZE/UV–vis, solution 1 at different stages of the experiment was injected in the UPLC/MS system. Four major peaks were detected: two peaks at *m/z* 359 and retention times of 7.5 and 8.1 min respectively, another peak eluting at 8.0 min and *m/z* 537, and a fourth peak co-eluting with this last compound at *m/z* 715.

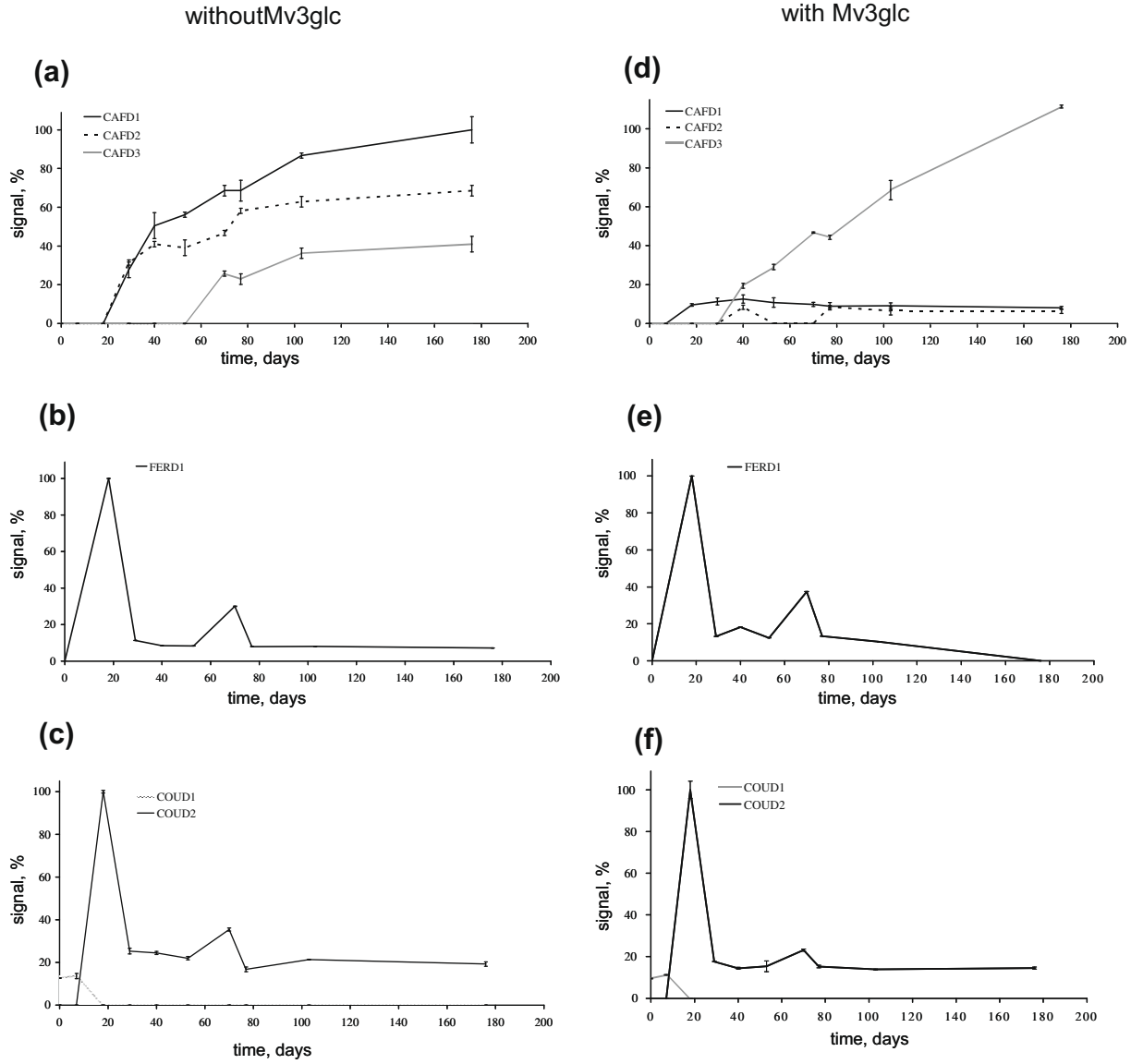

Fig. 1. Evolution of the derivatives formed in model solutions containing (a) caffeic acid (solution 1), (b) caffeic acid and Mvglc (solution 4), (c) ferulic acid (solution 2), (d) ferulic acid and Mvglc (solution 5), (e) *p*-coumaric acid (solution 3), and (f) *p*-coumaric acid and Mvglc (solution 6).

The first two derivatives detected by CZE/UV–vis, CAFD1 and CAFD2, could be assigned to isomers resulting from the condensation of two caffeic acids and formed between the oxidation product of the acid, a *ortho*-quinone, and caffeic acid (Fig. 2) (Singleton, 1987; Tulyathan, Boulton, & Singleton, 1987). Singleton (1987) described the formation of a dimer from the original phenol oxidised by the loss of two hydrogens but regenerated into reoxidizable hydroquinone form, also stating that it was not necessary to restrict this polymerisation reaction to semiquinone interaction alone and additional positional isomers could occur. Therefore, the formation of these two positional dimers (CAFD1 and CAFD2) could have been take place. The formation of these two dimers was confirmed by UPLC/MS as two derivatives at *m/z* 359 following identical evolution to that observed for CAFD1 and CAFD2 were detected. Moreover, the polymerisation reaction yielding derivatives CAFD1 and CAFD2 did not have to be restricted to one cycle (Singleton, 1987). Therefore, compound CAFD3 could be tentatively assigned to the condensation product of three caffeic acid units corresponding to the compound detected at *m/z* 537 and 8.0 min by means of UPLC/MS.

Despite the detection of a fourth compound by UPLC/MS at *m/z* 715 eluting at 8 min, corresponding to the condensation of four caffeic acids, it could not be detected by CZE/UV–vis, possibly due to the formation of low concentrations of this derivative and the higher sensitivity of the mass compared to the UV–vis detector.

The derivatives detected by CZE/UV–vis acquired lower charge-to-size ratios than caffeic acid (29.7 min) as they appeared at lower migration times; this could be explained by the fact that the increase in mass was higher than the increase in charges, providing all the derivatives with lower charge-to-size ratios than caffeic acid. Besides, the derivative CAFD3 could acquire more charges than CAFD1 and CAFD2 and therefore presented a higher charge-to-size ratio.

3.2. Study of ferulic acid solutions

In solution 2, which contained ferulic acid in synthetic wine, only one derivative was detected by means of CZE/UV–vis: FERD1, where FERD denotes ferulic acid derivative. This compound appeared at a migration time of 24.8 min in the electrophoregram, lower than ferulic acid (29.9 min).

The study of solution 2 (ferulic acid) by means of UPLC/MS indicated the presence of a compound following an evolution similar to CAFD1 and with *m/z* 387 (retention time = 7.4 min), concurring with the molecular ion mass given by the condensation of two ferulic acids. Although the formation of the *ortho*-quinone from ferulic acid was *a priori* difficult, and therefore the condensation described above for caffeic acid, oxidative reactions of phenolate ions take place through radicals (Cilliers & Singleton, 1990) and two semiquinone radicals can condense to form a dimer (Fulcrand, Hapiot, Neta, Pinson, & Rolando, 1997); this could explain the formation of the derivative detected: FERD1.

The derivative FERD1 was first detected after 7 days of reaction (Fig. 1b), and the highest concentration of the compound present in the solution was observed after 18 days of reaction, decreasing sharply thereafter.

Ferulic acid seems to be less reactive than caffeic acid since fewer derivatives are formed. Therefore, ferulic acid could be less involved in oxidation reactions and in the evolution of wines.

3.3. Study of p-coumaric acid solutions

Two derivatives were detected by CZE/UV–vis in solution 3: COUD1 and COUD2, where COUD denotes *p*-coumaric acid derivative. The derivative COUD1 was the first derivative detected with a migration time of 27.6 min, presenting lower charge-to-size ratios than the derivative COUD2 (29.3 min) and coumaric acid (36.3 min) as it migrated faster. Besides, COUD1 seemed to be very reactive or more unstable as it disappeared within the first days after formation (Fig. 1c).

The UPLC/MS injection of solution 3 indicated the presence of two isomers at *m/z* 327 and retention times of 7.2 and 7.3 min, respectively. The evolution study of the peak area detected by UPLC/MS for these two peaks permitted to compare their evolution with that of the electrophoretic peaks detected in solution 3; concluding that the evolution of derivatives COUD1 and COUD2 (detected by CZE/UV–vis) was similar to that found for the isomers at *m/z* 327 detected by UPLC/MS. Therefore, COUD1 and COUD2 could be tentatively assigned to dimers of *p*-coumaric acid with a molecular ion corresponding to 327.

Both derivatives, COUD1 and COUD2, presented lower charge-to-size ratios than the *p*-coumaric acid and could be assigned to polymerisation products with *m/z* 327 formed by the condensation of two semiquinone radicals deriving from the acid, as described above for the ferulic acid derivative, FERD1, formed in solution 2.

The derivatives COUD1 and COUD2 presented lower charge-to-size ratios than their acidic precursor, the *p*-coumaric acid, possibly suggesting that these derivatives increased more in size than the charges in comparison to *p*-coumaric acid. The compounds COUD2 and FERD1 seemed to be similar derivatives, following an identical evolution as shown in Fig. 1b and 1c.

Although in caffeic and *p*-coumaric acid solutions two positional isomers, CAFD1 and CAFD2, and COUD1 and COUD2 respectively, could have been formed by the condensation of two molecules of their acidic precursors, in ferulic acid solution only one derivative was detected (FERD1). This could be explained by

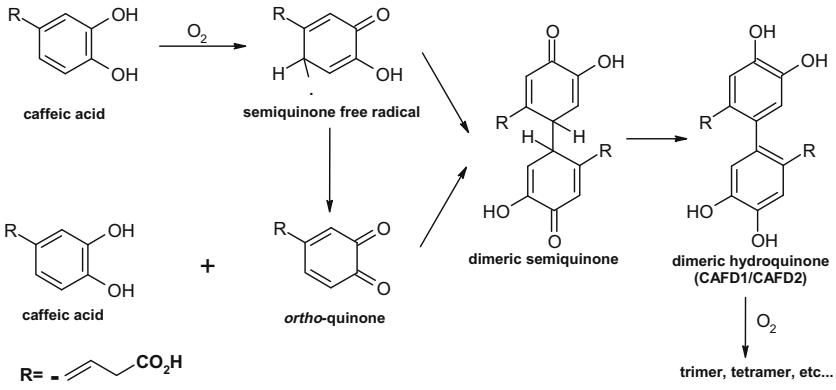

Fig. 2. A simplified example of the regeneration of caffeic acid by reaction between a quinone and caffeic acid.

the presence of an OCH_3- substituent in the ferulic acid moiety, which could restrict the formation of a positional isomer of FERD1 due to the larger size of this substituent in comparison with the OH- of caffeic acid and the H- of *p*-coumaric acid.

3.4. Study of caffeic acid and Mv3glc solutions

Six derivatives were detected in solution 4 by CZE/UV–vis. The following codes were used to name them: CAFD1, CAFD2, CAFD3, CAFD4, CAFD5, and CAFD6. Fig. 3a shows the electrophoregrams of solution 4 at three different stages of the study. Their migration times are listed in Table 1. Derivatives CAFD1, CAFD2 and CAFD3 were also detected in solution 1 in the absence of Mv3glc, the first two compounds being attributed to dimers of caffeic acid, whereas CAFD3 was tentatively assigned to the trimmer of this acid. The other three derivatives – CAFD4, CAFD5 and CAFD6 – formed in the presence of caffeic acid and Mv3glc. These compounds appeared at lower migration times than Mv3glc (19.9 min).

Fig. 1a and d show the evolution of the caffeic acid derivatives (CAFD1, CAFD2 and CAFD3) formed in absence and presence of the anthocyanin, respectively. As can be observed, the evolution of these compounds in the presence and absence of Mv3glc was different. The presence of the anthocyanin seemed to affect the evolution of caffeic acid in model solutions. Therefore, this different behaviour could also take place in white (without Mv3glc) and red wines (with Mv3glc) samples.

The UPLC/MS injection of solution 4 (Mv3glc and caffeic acid) at different stages of the experiment revealed the presence of six compounds, coinciding with the number of derivatives found by CZE/UV–vis (CAFD1-CAFD6). Three of these compounds corresponded to the condensation products of caffeic acid: CAFD1, CAFD2, and CAFD3 also detected in solution 1. A fourth derivative at *m/z* 669 and eluting at 8.3 min was detected. The study of the UPLC/MS peak area for this derivative was consistent with the evolution found for compound CAFD4 by means of CZE/UV–vis. This compound could therefore be tentatively assigned to malvidin

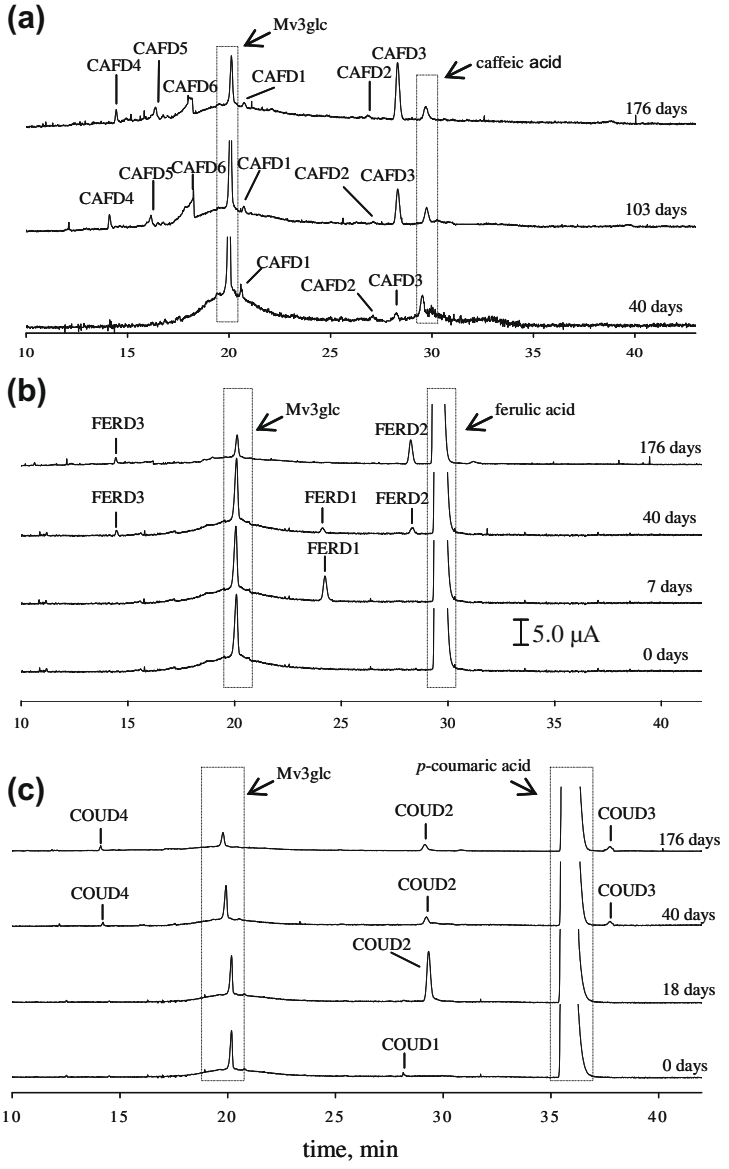

Fig. 3. Electrophoregrams at 280 nm of model solutions containing (a) caffeic acid and Mvglc (solution 4), (b) ferulic acid and Mvglc (solution 5), and (c) *p*-coumaric acid and Mvglc (solution 6).

3-glucoside 4-vinylsyringol; this derivative has been reported previously (Schwarz et al., 2003a), and its formation was explained by the direct reaction between syringic acid and the anthocyanin. The presence of small amounts of a peak at *m/z* 199 in solution 4 could explain the presence of syringic acid (not detectable by CZE/UV–vis), and consequently the formation of malvidin 3-glucoside 4-vinylsyringol. Syringic acid would have resulted from the moiety corresponding to the B-ring released after degradation of Mv3glc (Piffaut, Kader, Girardin, & Metche, 1994; Santos-Buelga, Bravo-Haro, & Rivas-Gonzalo, 1995).

Another compound detected by UPLC/MS presented a molecular ion at *m/z* 803 and retention time of 7.9 min attributable to derivative CAFD5; although it has not been reported before, this derivative can be tentatively assigned to the structure of a Pinotin A linked to a caffeic acid as shown in Fig. 4.

A derivative with a molecular ion at *m/z* 625 and retention time of 8.0 min was also detected by the UPLC system. The study of the UPLC/MS peak area for this derivative during the experiment was consistent with the evolution observed for compound CAFD6; this peak could therefore be tentatively assigned to malvidin 3-glucoside 4-vinylcatechol (Pinotin A). The formation of this compound in red wines (Rentzsch et al., 2007; Schwarz, Picazo-Bacete, Winterhalter, & Hermosín-Gutierrez, 2005) and model solutions (Schwarz et al., 2003a; Gómez-Miguez et al., 2006; Schwarz, Jerz, & Winterhalter, 2003b) has been previously reported.

The derivatives CAFD4, CAFD5, and CAFD6 presented red/violet colour with maximum absorbance in the blue region, at 583.5 for CAFD6 and CAFD5 (Fig. 5a), and 590 for CAFD4 (Fig. 5b). These findings were consistent with the studies carried out on hydroxycinnamyl-derived portisins by Oliveira et al. (2007), who observed a bathochromic shift in the λ_{max} of these compounds at basic pH values (9.0 and 11.0) probably corresponding to the equilibrium displacement toward the formation of the quinoidal forms of these pigments. This behaviour was also expected for the spectral characteristics of CAFD4, CAFD5, and CAFD6 at the pH values (9.4) of the CZE-buffer since a malvidin moiety was present in their structures.

Fig. 6a studies the evolution of Mv3glc and the red derivatives formed from caffeic acid and the anthocyanin. The red derivative CAFD6, with the highest charge-to-size ratio, was the derivative formed in the highest proportion, whereas the other two derivatives seemed to form in a similar proportion related to Mv3glc. Compound CAFD5 seemed to be stable or at least it displayed less reactivity than CAFD4 because once it formed it remained constant.

The behaviour of compounds CAFD5, and CAFD6 coincided with that reported in literature, where the formation of anthocyanin-vinylphenols by direct reaction of hydroxycinnamic acids and anthocyanins has been described to constantly increase during storage of model solutions (Schwarz et al., 2003a) and therefore in wines as long as free anthocyanins and cinnamic acids are available.

All this prompts the conclusion that three derivatives were formed in solution containing Mv3glc and caffeic acid and could tentatively be attributed to malvidin 3-glucoside 4-vinylcatechol (Pinotin A), malvidin 3-glucoside 4-vinylsyringol, and a Pinotin A derivative.

3.5. Study of ferulic acid and Mv3glc solutions

Fig. 3b shows the electrophoregrams registered for solution 5 during the experiment. Three derivatives were detected: FERD1, FERD2, and FERD3, whose migration times are shown in Table 1. The compound FERD1 derived from ferulic acid as it appeared in solution 2 in the absence of Mv3glc, at migration times and UV–vis spectra comparable to those of the derivative formed in this solution and tentatively identified as the dimer of ferulic acid.

Fig. 1b and 1e represented the evolution of the derivative FERD1 in solution 2 and 5, respectively, related to ferulic acid over time, and in contrast to what occurred in the case of caffeic acid, the evolution of this derivative was similar in the presence and absence of Mv3glc, although it disappeared in the presence of Mv3glc and after 176 days.

The second derivative detected in solution 5, FERD2, formed in the presence of ferulic acid and Mv3glc. In an attempt to identify this compound, solution 5 (Mv3glc and ferulic acid) at the different stages of the experiment was injected in the UPLC/MS system. The

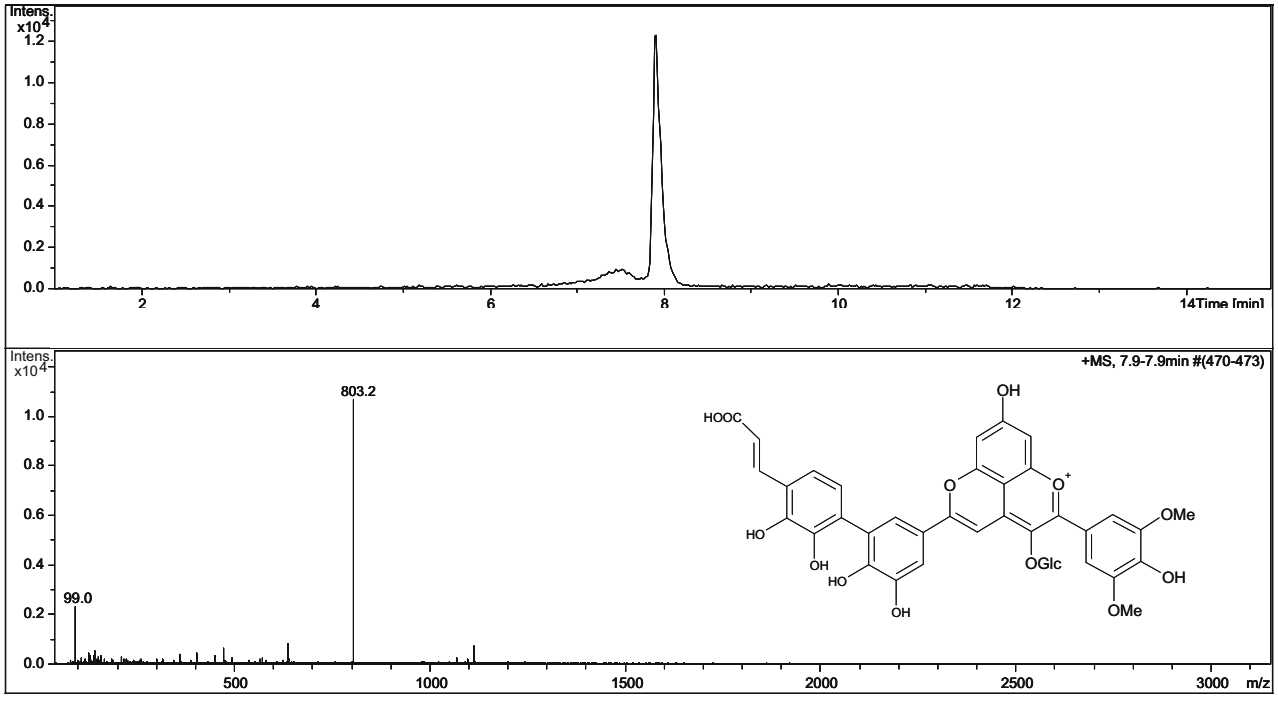

Fig. 4. UPLC chromatogram (extracted ion), and mass spectrum of derivative CAFD5.

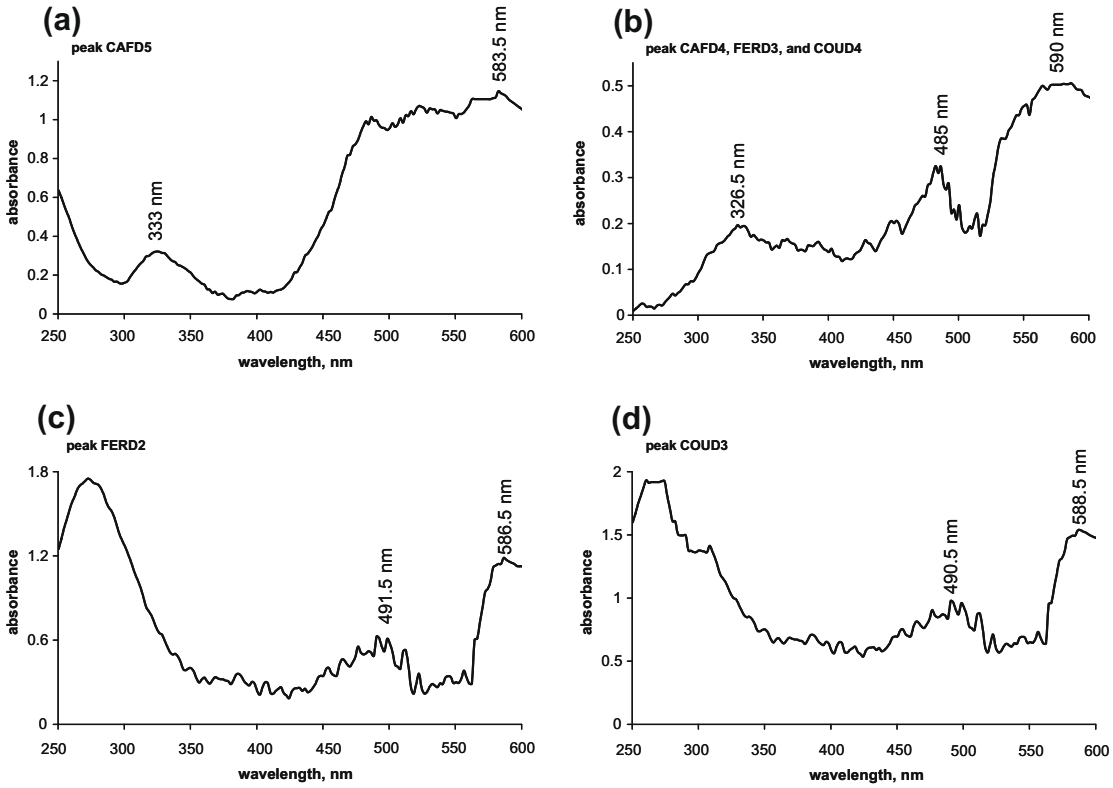

Fig. 5. UV–vis spectra of peaks: (a) CAFD5, (b) CAFD4, FERD3, and COUD4, (c) FERD2, and (d) COUD3.

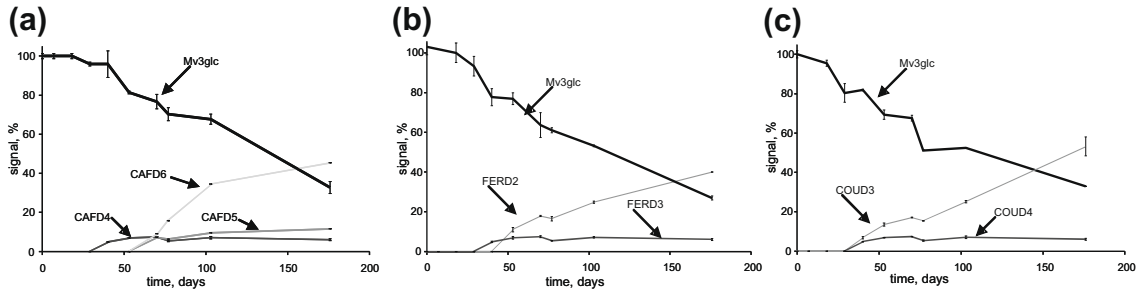

Fig. 6. Evolution of the derivatives of Mv3glc and (a) caffeic acid, (b) ferulic acid, and (c) *p*-coumaric acid.

chromatogram revealed the presence of one derivative eluting at 7.7 min and with a molecular ion at *m/z* 639 following similar evolution to FERD2. This compound could be tentatively assigned to the derivative malvidin 3-glucoside 4-vinylguaiacol. The formation of this compound in red wines (Dugelay, Gunata, Sapis, Baumes, & Bayonove, 1993; Hayasaka & Asenstorfer, 2002), and model solutions (Schwarz et al., 2003a) has been reported previously and as in the case of caffeic acid this pyranoanthocyanin could be formed by direct reaction between the intact ferulic acid and Mv3glc.

The derivative FERD2 had a red/violet colour as it absorbed in the visible region with a maximum at 586.5 (Fig. 5c); the same finding was also observed for malvidin and caffeic acid derivatives and could be attributed to the equilibrium displacement of these pigments toward the formation of quinoidal forms. However, the spectrum of FERD2 was slightly bathochromically displayed compared with the pinotin derivatives (CAFD5 and CAFD6) formed in the presence of the anthocyanin and caffeic acid found in solution 4.

Fig. 6b shows the evolution in solution 5 of derivative FERD2, tentatively assigned to malvidin 3-glucoside 4-vinylguaiacol, and Mv3glc. This derivative was first detected after 50 days of reaction and it formed until the end of the experiment. This finding was consistent with data obtained in this study for caffeic acid and in previous studies carried out by Schwarz et al. (2003a), where these pigments were reported to be potentially attractive for use as ageing indicators for red wines as their concentration would constantly increase during ageing. A derivative at *m/z* 669 and eluting at 8.3 min was also detected by UPLC/MS. Migration and retention times, as well as mass and UV–vis spectra (Fig. 5b) and its identical evolution in solutions 4, and 5 of FERD3 were all comparable to those of CAFD4. Therefore, FERD3 was tentatively assigned to malvidin 3-glucoside 4-vinylsyringol, formed by direct reaction between the syringic acid and the anthocyanin.

3.6. Study of solutions of p-coumaric acid and Mv3glc

Four derivatives were detected in solution 6; two of them, COUD1 and COUD2, deriving from *p*-coumaric acid, were also detected in solution 3. The presence of Mv3glc did not seem to affect the behaviour of *p*-coumaric acid derivatives since both presented similar evolution rates as shown in Fig. 1c and 1f. Another two derivatives, COUD3 and COUD4, migrated at 37.9 and 14.5 min,

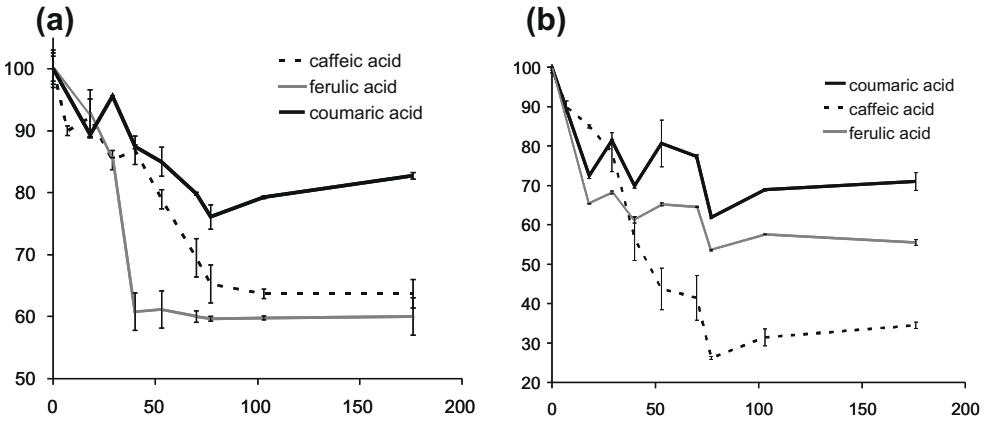

Fig. 7. Evolution of hydroxycinnamic acids (a) without Mv3glc and (b) with Mv3glc.

respectively. Fig. 5d shows the UV–vis spectra of COUD3 which presented a maximum of absorption at 588.5 nm.

Derivative COUD3 was formed in the presence of *p*-coumaric acid and Mv3glc, and in an attempt to identify this compound, solution 6 was injected in the UPLC/MS system. The chromatograms revealed the presence of one derivative eluting at 7.9 min with a molecular ion at *m/z* 609 and presenting similar evolution to that COUD3. This compound could be tentatively assigned to the derivative malvidin 3-glucoside 4-vinylphenol. This derivative has already been reported to form in model solutions of the hydroxycinnamic acid and Mv3glc (Schwarz et al., 2003a; Gómez-Miguez et al., 2006; Schwarz et al., 2003b).

The derivative COUD3 was detected after 40 days of reaction (Fig. 6c) and continued to form afterwards; this concurred with the evolution of pyranoanthocyanins directly formed between caffeic acid or ferulic acid and an anthocyanin and also reported by Schwarz et al. (2003a).

It is important to highlight those derivative COUD4 properties, migration and retention times, UV–vis (Fig. 5b) and mass spectra as well as its evolution coincided with those of FERD3 and CAFD4, and it could therefore be assigned to the malvidin and syringic acid derivative, malvidin 3-glucoside 4-vinylsyringol.

3.7. Evolution of hydroxycinnamic acids in the presence and absence of Mv3glc

The evolution of free caffeic, ferulic, and *p*-coumaric acids in model solutions presented significant differences. Fig. 7a and 7b show the evolution of the acids in the absence and presence of Mv3glc. Two stages could be differentiated: the first, where acids decreased more rapidly, especially ferulic acid in solutions without the anthocyanin and caffeic acid in the presence of Mv3glc; and a second stage where acid concentrations displayed a slight variation. In the second stage, hydroxycinnamic acids increased in the presence of the anthocyanin; this could be explained by the regeneration of the acids through coupled oxydoreduction reactions. However, in the absence of Mv3glc this regeneration was only observed for *p*-coumaric acid. Therefore, if the stability of wine colour depends on these compounds, the presence of free caffeic, ferulic and *p*-coumaric acids would imply the stabilisation of colour matter in red wines.

4. Conclusions

The electrophoretic behaviour of Mv3glc and hydroxycinnamic acid derivatives, as well as their evolution in model solutions, has been studied by CZE/UV–vis. The UPLC/MS technique also enabled the tentative identification of the derivatives formed in samples.

The formation of new derivatives of Mv3glc and caffeic, ferulic and *p*-coumaric acids was confirmed. In solutions containing malvidin 3-*O*-glucoside together with caffeic acid, six derivatives were found: three condensation products of caffeic acid, 4-vinylcatechol, 4-vinylsyringol, and a compound with a molecular ion at *m/z* 803, whose structure was tentatively assigned to a caffeic acid linked to Pinotin A. The derivatives 4-vinylphenol and 4-vinylguaiacol pyranoanthocyanins were detected in solutions containing malvidin 3-*O*-glucoside together with *p*-coumaric and ferulic acid, respectively, whereas the derivative 4-vinylsyringol was detected in both solutions.

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