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Advanced ultra high pressure liquid chromatography–tandem mass spectrometric methods for the screening of red wine anthocyanins and derived pigments

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ABSTRACT

Anthocyanins are responsible for the colour of red grapes and wine. In addition to their contribution to the sensory properties of wine, these compounds are also of interest due to their beneficial biological properties. Wine anthocyanins exhibit a large structural diversity due to variations in glycosylation and acylation patterns, which is further exacerbated by the diverse reactions involving grape-derived anthocyanins during wine ageing. Chromatographic as well as mass spectrometric resolution of wine anthocyanins is often precluded due to the complexity of these compounds. In this paper we report a rapid, high-efficiency ultra high pressure liquid chromatography (UHPLC) procedure with tandem mass spectrometric (MS/MS) detection for the in-depth screening of wine pigments. Selective detection of wine anthocyanins and derived pigments was achieved utilizing MS/MS in neutral loss scanning mode to observe the loss of dehydrated sugar moieties. This facilitated tentative compound identification based on molar mass information as well as the structured elution order of these compounds. In a second experiment, product ion spectra were recorded to allow identification of the anthocyanidin base using characteristic fragmentation patterns. The proposed methodology therefore involves two analyses for the sensitive and accurate identification of anthocyanins and their derived products in red wines. Mass spectra of wine anthocyanins under high energy collision induced dissociation (CID) conditions are reported, some for the first time. Significantly, chemical alteration of anthocyanins during wine ageing results in an off-set of the predominant fragments for each anthocyanidin base, whilst maintaining similar relative intensities. This allows unambiguous assignment of the derived products of anthocyanidin-glycosides. Using this approach, a total of 121 anthocyanins and derived compounds were identified in wines based on their relative reversed phase elution order as well as mass spectral information.

1. Introduction

The colour of red grapes and wine is attributed largely to the anthocyanins (anthocyanidin-glycosides). Apart from their important role in the aesthetic perception and quality of red wines, anthocyanins may also serve as markers for wine authentication and possess beneficial biological properties, which have led to increasing recent interest in their analytical determination [1,2].

Anthocyanins belong to the flavonoid family and exist as glycosides through conjugation of anthocyanidins with primarily glucose. These compounds exhibit a large structural diversity due to the substitution pattern of the anthocyanidin base as well as variations in the position, number and acylation of sugar moieties. In *Vitis vinifera* varieties, pigments are mainly in the 3-O-glucoside form, whereas in other *Vitis* species 3,5-diglucosides also occur. Five anthocyanidin bases are present in red grapes namely malvidin, delphinidin, peonidin, cyanidin and petunidin. Malvidin-3-O-glucoside is the predominant pigment in *V. vinifera* varieties [2–4]. During maturation and ageing of red wines, anthocyanins undergo addition reactions with other wine constituents to yield pigments that impart important changes to the product, notably in terms of colour and taste. They may react with flavanols, either directly or mediated by other compounds such as acetaldehyde [5–8]. Wine constituents possessing a polarizable double bond (such as pyruvic acid [9,10], acetaldehyde [11] or vinylphenol [12]) also undergo cyclo-addition reactions with anthocyanins to add a pyran ring to the anthocyanidin base [5,7,9,10]. The pyranoanthocyanins possess a tawny colour and are more stable to temperature and pH effects as well as to sulphur dioxide bleaching [3,13,14]. Pyranoanthocyanins may further react with other

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wine constituents such as vinyl-flavanols to produce blue pigments named portisins [15]. All of these anthocyanin-derived pigments contribute to the observed change in wine colour from the initial purple-red to a brick-red hue characteristic of aged wines and there is a concurrent decrease in astringency due to the involvement of tannins in these processes [13].

Reversed phase liquid chromatography (RP-LC) is the most widely used separation technique for the study of anthocyanins. These compounds are subject to pH dependent inter-conversions between various molecular forms and a highly acidic mobile phase (pH < 2) is therefore required to ensure that they are maintained predominantly in the flavylium cationic form for maximum chromatographic efficiency [2]. However, even at low pH some inter-conversion between the anthocyanin flavylium cationic and carbinol pseudobasic forms occurs [16]. The slow rate of this inter-conversion reaction dictates relatively low optimal mobile phase velocities (~0.1 mm/s) for their separation. Chromatographic efficiency may further be improved by using small-particle columns (~1.7 μm) and elevated temperatures (~50 °C) to increase the rate of inter-conversion reactions as well as mass transfer [16]. de Villiers et al. [16] demonstrated that thermal degradation of anthocyanins is avoided at this temperature, whilst gradient separation on 1.7 μm phases at elevated temperature provides highly efficient separation of wine anthocyanins [17].

Diode array detection (DAD) and mass spectrometry (MS) or tandem mass spectrometry (MS/MS) are the most widely used chromatographic detection techniques for the study of anthocyanins. LC–DAD is inherently suited to provide information on the colour of these compounds and can also tentatively distinguish between the main phenolic structures, since these display unique ultraviolet–visible (UV–vis) absorption spectra characterized by absorption in the visible range around 500 nm. Mass spectrometry provides benefits such as increased sensitivity and structural information compared to LC–DAD. Various MS instruments have been applied to anthocyanin analysis, varying between simple quadrupole- and more advanced tandem and high-resolution instruments such as triple quadrupole and quadrupole-time-of-flight (Q-TOF) systems. Tandem mass spectrometry is particularly suited for structure elucidation and compound identification since information pertaining to the aglycone moiety, type and number of sugars and other substituents can be obtained [1]. Since few reference standards for anthocyanins are available (in particular, no standards are available for derived wine anthocyanins), compound identification is often tentative and is usually based on RP-LC elution order, UV–vis spectra as well as mass spectral information (molecular weight, accurate mass and/or MS/MS fragmentation [1]). However, due to the large numbers and structural similarity of these compounds, complete chromatographic separation of wine anthocyanins is not attainable. Moreover, compound identification is complicated by the fact that some non-anthocyanin phenolic compounds have similar mass spectral characteristics.

Direct infusion MS has also been described for the study of these compounds [14,18]. These techniques use direct introduction of purified sample extracts, producing a rapid and sensitive methodology for qualitative and semi-quantitative determination of anthocyanins. However, a number of anthocyanins and derived products have identical mass spectral properties, which render them indistinguishable by MS alone. In contrast, chromatographic separation in combination with MS provides adequate resolution to identify these compounds with higher accuracy and may also reduce possible ionization suppression effects known to be associated with electrospray ionization. However, since complete chromatographic resolution is not ensured, it is imperative that mass spectral information, in addition to the molecular ion and mass of the sugar moiety, is available for unambiguous compound identification.

In this study, a methodology is proposed for the analysis and unambiguous identification of the principal anthocyanins and derived compounds in red wines. An ultra high pressure liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method utilizing optimized chromatographic parameters [16,17] was developed to investigate these compounds in *V. vinifera* cv. Pinotage wines. The anthocyanins and anthocyanin-derived compounds are detected with great selectivity using tandem mass spectrometry in neutral loss scanning mode. By performing concurrent product ion scans, the aglycone cation moieties of each detected compound may be identified unambiguously via their characteristic fragmentation patterns under high collision energy conditions [14]. For each detected compound, identification is therefore based on the well-known RP-LC elution order, molecular weight of the compound and mass of sugar moiety (obtained in the neutral loss experiment) as well as identification of the aglycone cation (via spectral information yielded by the survey scan experiment). Three families of anthocyanin derived pigments were investigated in this study, namely anthocyanins, pyranoanthocyanins and flavanol-anthocyanin condensation products.

2. Experimental

2.1. Materials

Malvidin-3,5-diglucoside was purchased from Sigma–Aldrich (Mulbarton, South Africa), malvidin-3-glucoside chloride (MVG, Oenin chloride) was obtained from Extrasynthese (Genay, France) and cyanidin-3-O-glucoside from Polyphenols Laboratories (Sandnes, Norway). Standards were prepared by weighing reference material on an analytical balance and all dilutions were made using volumetric glassware in a solution of 10% ethanol in water. All other chemicals and solvents were HPLC grade and were supplied by Sigma–Aldrich (Mulbarton, South Africa).

2.2. Samples

South African wines were commercial products of the cultivar *V. vinifera* cv. Pinotage, aged between 1 and 4 years. Two German Dornfelder hybrid-cultivar wines were also studied since these contain higher levels of diglucoside anthocyanins. The Dornfelder wines were obtained from import submissions (South African Department of Agriculture, Forestry and Fisheries). Samples were filtered using 0.22 μm HVLP syringe filters (Millipore, Milford, MA, USA) prior to analysis.

2.3. UHPLC–MS/MS analysis

A Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA) incorporating a binary pump, vacuum degasser, autosampler, column oven and Micromass Xevo tandem quadrupole mass spectrometric detector (Manchester, UK) equipped with ESI probe was used. Reversed phase separation was performed using an Acquity BEH C18 column (2.1 mm × 100 mm, 1.7 μm particle size) at a temperature of 50 °C. Mobile phase A was 7.5% formic acid in water and mobile phase B acetonitrile. The gradient started with 1% B isocratically for 0.5 min followed by a linear increase to 15% at 15 min, 23% at 20 min and 28% at 25 min. Column clean-up at 100% B then followed for 1 min followed by re-equilibration for 4 min (total run-time of 30 min). The flow-rate was 0.1 mL/min throughout and injection volumes of 10 μL were used. The source conditions were as follows: capillary voltage 2.5 kV, cone voltage 15 V, whilst the desolvation temperature was 500 °C and the desolvation- and cone gas (both nitrogen) flows were 1000 and 50 L/h, respectively. Two different MS data acquisition modes were used. Firstly, the instrument was operated in neutral loss mode, monitoring 4 neutral loss

Table 1
Chromatographic and mass spectrometric parameters of detected anthocyanidin-glucosides, -diglucosides and oligomeric anthocyanins.

Peak no[a]	Compound[b]	Retention time (min)	M^+ (m/z)	Neutral loss offset (amu)
13	Dp-3-O-glucoside	9.29	465	162
16	Cy-3-O-glucoside	11.00	449	162
23	Pt-3-O-glucoside	12.12	479	162
34	Pe-3-O-glucoside	13.97	463	162
41	Mv-3-O-glucoside	14.75	493	162
42	Dp-3-O-acetylglucoside	15.08	507	204
52	Cy-3-O-acetylglucoside	17.02	491	204
60	Pt-3-O-acetylglucoside	17.84	521	204
71	Pe-3-O-acetylglucoside	19.72	505	204
75	Mv-3-O-acetylglucoside	20.16	535	204
68	Dp-3-O-coumaroylglucoside (*cis*-isomer)	18.87[c]	611	308
69	Dp-3-O-coumaroylglucoside (*trans*-isomer)	18.99[c]	611	308
79	Cy-3-O-coumaroylglucoside (*cis*-isomer)	20.37	595	308
81	Cy-3-O-coumaroylglucoside (*trans*-isomer)	20.49	595	308
86	Pt-3-O-coumaroylglucoside (*cis*-isomer)	20.78	625	308
87	Pt-3-O-coumaroylglucoside (*trans*-isomer)	20.91	625	308
90	Pe-3-O-coumaroylglucoside (*cis*-isomer)	21.51	609	308
97	Pe-3-O-coumaroylglucoside (*trans*-isomer)	22.27	609	308
92	Mv-3-O-coumaroylglucoside (*cis*-isomer)	21.76	639	308
98	Mv-3-O-coumaroylglucoside (*trans*-isomer)	22.48	639	308
56	Dp-3-O-caffeoylglucoside	17.26[c]	627	324
66	Pt-3-O-caffeoylglucoside	18.85	641	324
80	Pe-3-O-caffeoylglucoside	20.37[c]	625	324
83	Mv-3-O-caffeoylglucoside	20.65[c]	655	324
9	Dp-3,5-di-O-glucoside	7.93[c]	627	324
18	Pt-3,5-di-O-glucoside	11.08[c]	641	324
21	Pe-3,5-di-O-glucoside	11.76[c]	625	324
25	Mv-3,5-di-O-glucoside	12.36[c]	655	324
10	Dp-3,7-di-O-glucoside	8.70[c]	627	324
24	Pt-3,7-di-O-glucoside	12.28[c]	641	324
26	Pe-3,7-di-O-glucoside	12.73	625	324
30	Mv-3,7-di-O-glucoside	13.51	655	324
35	Pe-5,7-di-O-glucoside	14.06[c]	625	324
45	Mv-5,7-di-O-glucoside	15.13	655	324
12	Dp-3-O-galactoside	9.09	465	162
33	Pe-3-O-galactoside	13.84	463	162
39	Mv-3-O-galactoside	14.45	493	162
61	Mv-Mv-2-O-glucoside dimer	17.85[c]	985	162/324
76	Mv-O-glucoside-Mv-O-acetylglucoside dimer	20.18[c]	1027	204

[a] Peak numbers correspond to Fig. 2.
[b] Dp, delphinidin; Cy, cyanidin; Pt, petunidin; Pe, peonidin; Mv, malvidin.
[c] Survey scan spectra do not allow conclusive identification of aglycone due to low signal intensity.

functions: m/z 162, 204, 308 and 324. The mass range for the first 3 neutral losses was m/z 400–900, and m/z 430–1200 for the 324 neutral loss scan. The collision energy was 25 V for neutral loss experiments. Secondly, 4 methods were used to obtain product ion spectra for the peaks detected in the neutral loss experiments for qualitative information. For this purpose, 4 separate survey scan methods were used for each of the 4 neutral losses (m/z 162, 204, 308 and 324), and the instrument was set at a low trigger sensitivity in each case. In this mode, the instrument switches from neutral loss mode to product ion scan mode using a scan range of m/z 100–1500 at a collision energy of 50 V. The limit of detection (LOD) of the neutral loss method was ~0.1 mg/L, based on a signal-to-noise ratio of 3:1 determined for malvidin-3-O-glucoside. The survey scan method yielded consistent qualitative information of the aglycone cations at concentrations above 5 mg/L, as determined for malvidin-3-O-glucoside.

2.4. High resolution MS analysis

A Waters Synapt G2 quadrupole-time-of-flight (QTOF) mass spectrometer was used for high resolution LC–MS/MS analysis to confirm the molecular formulas of fragment ions. The LC method and source conditions were identical to those described above for UHPLC–MS/MS analysis except that a collision energy of 60 V was used. Leucine encaphalin (m/z = 556.2771) was used as reference (lock) mass and the instrument was calibrated with sodium formate. The resolution of the instrument is 20,000–24,000 in the mass range that data was acquired in and the mass accuracy was better than 2 ppm.

3. Results and discussion

Direct infusion mass spectrometry provides a rapid and sensitive methodology for qualitative and semi-quantitative determination of anthocyanins. However, a number of wine anthocyanins have identical mass spectral properties and can therefore not be distinguished utilizing mass spectrometry exclusively. For example, the isomers of the 3-O-coumaroylglucosides and 3-O-caffeoylglucosides cannot be differentiated by mass spectrometry, whilst the 3-O-caffeoylglucosides have identical molecular ion masses, aglycone cations and sugar moiety masses compared to the corresponding 3,5- and 3,7-diglucosides. In addition, several anthocyanin-derived wine pigments have identical masses and primary fragmentation patterns, such as the vinylformic acid adduct of cyanidin-3-glucoside and the acetaldehyde adduct of mavidin-3-glucoside (m/z 517, loss of 162), the 4-vinylguaicol adduct of peonidin-coumaroylglucoside and the 4-vinylphenol adduct of malvidin-coumaroylglucoside (m/z 755, loss of 308), and the 4-vinylcatechol adduct of malvidin-glucoside and the 4-vinylguaicol adduct of petunidin-glucoside (m/z 609, loss of 162) (see further).

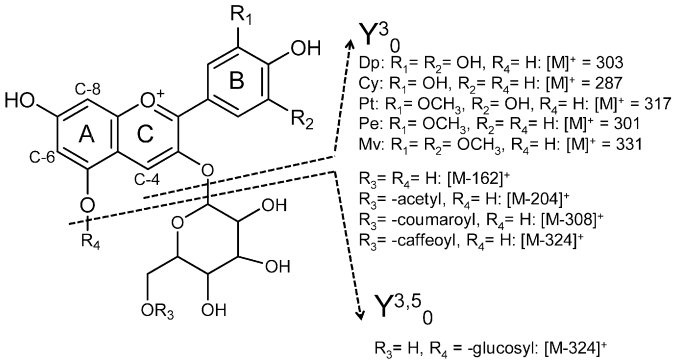

Fig. 1. Neutral loss fragmentation of the anthocyanins and derived products under low energy CID conditions (25 V). Dp, delphinidin; Cy, cyanidin; Pt, petunidin; Pe, peonidin; Mv, malvidin.

This renders these compounds indistinguishable by mass spectrometry alone. Since these isobaric compounds can be separated chromatographically, RP-LC in combination with mass spectrometry offers a much more accurate technique for the detailed analysis of anthocyanins.

The chromatographic method used here is based on the optimal kinetic configuration for the RP-LC analysis of anthocyanins [16,17]. Due to the unique chromatographic behaviour of anthocyanins [16], very low optimal linear velocities are required for improved chromatographic separation of these molecules. By using a column packed with 1.7 μm particles operated at elevated temperature and the optimal flow rate for anthocyanins, the high-efficiency analysis of these compounds within reasonably short analysis times is possible [17] (see Fig. 2 further).

LC–MS in scan mode has been used extensively for the screening of wine anthocyanins, although compound identification is often tentatively based on elution order and molecular ion information only [17,19,20]. On the other hand, tandem mass spectrometry is commonly used for highly selective and sensitive detection of a limited number of target analytes only [21,22].

Glycosylated anthocyanins and their derivatives are known to fragment via the loss of the dehydrated sugar moiety [1,14]. This phenomenon may be exploited to allow highly selective detection of these compounds by tandem mass spectrometry in neutral loss scanning mode. This mode involves both quadrupole analysers being scanned with a preset mass offset so that when a target primary ion loses the predetermined mass, a signal is generated to detect that compound exclusively. Monitoring selected neutral losses in this manner allows the selective detection of anthocyanin-glycosides (neutral loss *m*/*z* 162), -diglucosides (neutral loss *m*/*z* 324), -acetylglucosides (neutral loss *m*/*z* 204), -coumaroylglucosides (neutral loss *m*/*z* 308) and caffeoylglucosides (neutral loss *m*/*z* 324), as well as the corresponding derived condensation products formed during wine production. The highly selective nature of neutral loss MS/MS experiments simplifies chromatographic analysis and facilitates tentative compound identification utilizing RP-LC chromatographic elution order. The neutral loss experiment also yields information pertaining to the molecular mass and mass of sugar moiety of the detected compounds. However, as highlighted above, the fact that various wine constituents have identical molecular ion masses and fragmentation patterns means that unambiguous compound identification is not guaranteed using this approach. By performing a second experiment in survey scan mode, the cationic aglycone of each detected compound may be identified by its characteristic fragmentation pattern, as described by Hayasaka and Asenstrofer [14]. In survey scan mode, the instrument performs neutral loss scanning and switches to product ion mode at a specified ion count

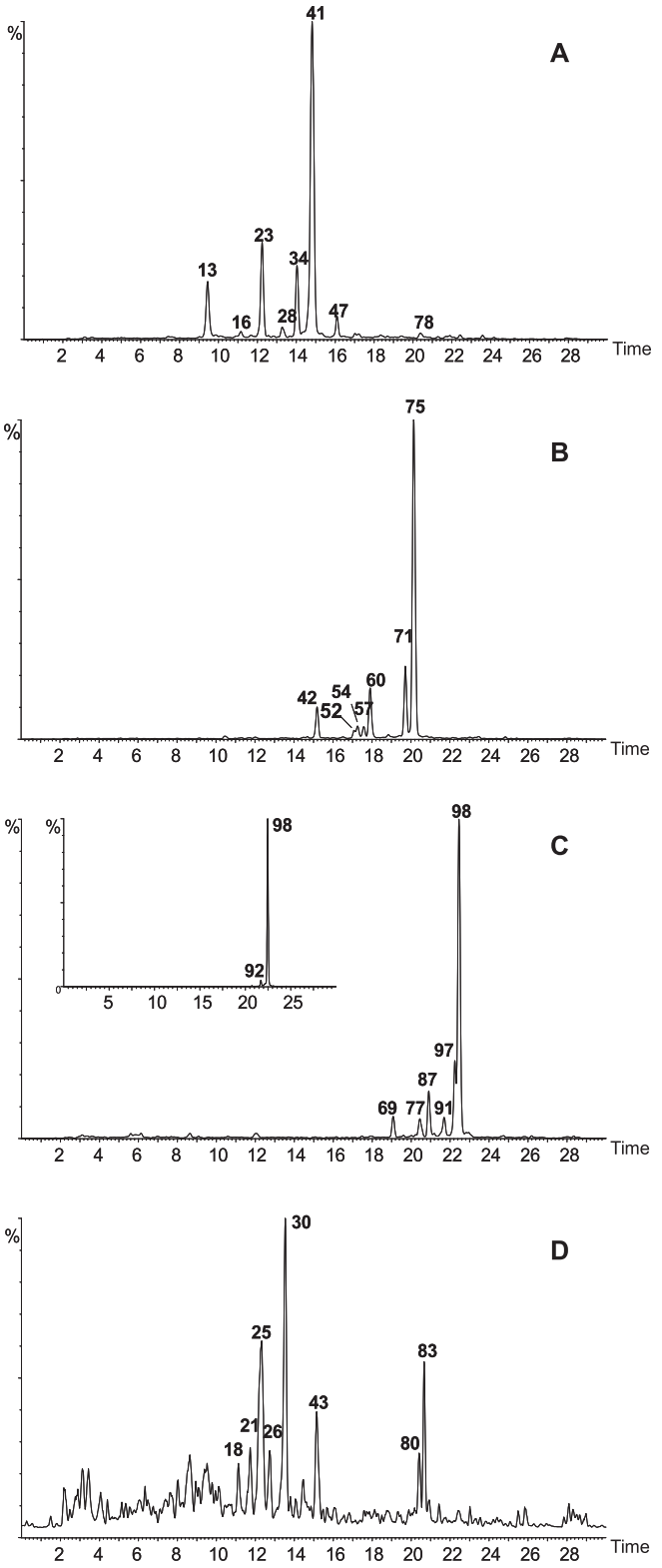

Fig. 2. TICs of the LC–MS/MS neutral loss experiments recorded at (A) 162 amu (elimination of glucose), (B) 204 amu (elimination of acetylglucose), (C) 308 amu (elimination of *p*-coumaroylglucose) and (D) 324 amu (elimination of caffeoylglucose as well as diglucosides) for the analysis of a 2010 Pinotage wine. Inset (C): neutral loss chromatogram (308 amu) showing the isomers of malvidin-3-O-coumaroylglucoside (extracted ion at *m*/*z* = 639, *cis*-isomer at 21.46 min and *trans*-isomer at 22.48 min). Peak numbers correspond to Tables 1 and 3.

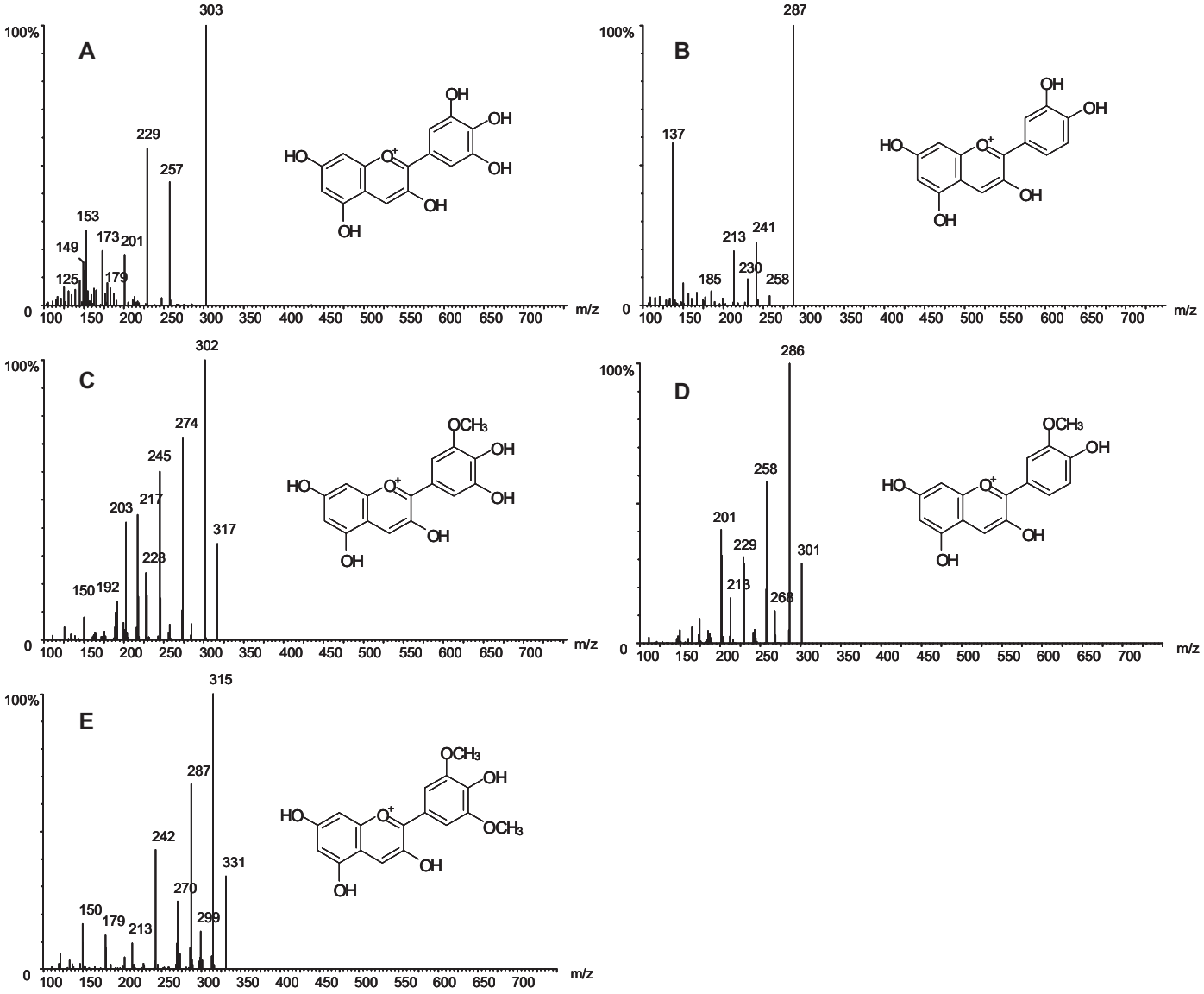

Fig. 3. Characteristic product ion mass spectra of the anthocyanidin bases obtained in survey scan mode (concurrently with detection of the 3-O-monoglucoside anthocyanins in neutral loss mode at 162 amu) for (A) delphinidin, (B) cyanidin, (C) petunidin, (D) peonidin and (E) malvidin.

to fragment the target ion. The combination of chromatographic separation with MS/MS detection in neutral loss mode, combined with characterization of the relevant aglycone by means of product ion spectra, significantly enhances the certainty in compound identification. This is especially relevant for wine pigments, since standards for these compounds are not commercially available and therefore comparison of retention time and mass spectral data with authentic standards is not possible. Using this approach, we were able to identify 121 anthocyanins, pyranoanthocyanins and anthocyanin-flavanol adducts in Pinotage wines. The identification of each of these classes of compounds are discussed categorically in terms of chromatographic and MS/MS information in the following sections.

3.1. Anthocyanins

Fig. 1 shows the fragmentation patterns for anthocyanins under low energy collision induced dissociation (CID) conditions. The nomenclature used here to discuss the fragmentation is in accordance with the adapted approach reported by Abad-García et al. [1]: $Y_c^{a,b}$ indicates the aglycone ion, where the glycosidic bond is specified by the subscript c (i.e. 0 for the linkage with the aglycone, 1 for the inter-glycosidic bond of diglycosides). The position of glycosylation is denoted by superscripts a and b. Under high collision energies, rupture of the C-ring bonds results in charged fragments containing the A- and B-rings, denoted $[^{c,d}A]^+$ and $[^{c,d}B]^+$, respectively, where the position of the C-ring fragmentation is specified by the superscripts c and d (see Fig. 4 further).

Wine anthocyanins comprise mainly the anthocyanidin-monoglucosides and their acetyl-, *p*-coumaroyl-, and caffeoyl-derivatives, as well as two groups of di-glucosides. Table 1 summarizes the chromatographic and mass spectral parameters of the anthocyanins detected in Pinotage wines. These compounds display a structured elution pattern based on the characteristics of the anthocyanidin base [1]. The neutral loss chromatograms obtained for 162 amu (3-O-monoglucosides), 204 amu (3-O-acetylglucosides), 308 amu (3-O-coumaroylglucosides) and 324 amu (diglucosides and 3-O-caffeoylglucosides) (Fig. 2) clearly demonstrate this structured elution order. Within each group, the elution order is: delphinidin < cyanidin < petunidin < peonidin < malvidin. This elution order is a function of the polarity of attached functional groups to

the anthocyanidin base (hydroxyl groups decrease retention whereas methoxyl groups increase retention under RP-LC conditions). The acylated anthocyanins elute after the anthocyanins, whilst retention increase with hydrophobicity. Therefore the elution order is 3-O-monoglucosides < 3-O-acetylglucosides < 3-O-coumaroylglucosides, whilst the 3-O-caffeoylglucosides eluted amongst the 3-O-acetylglucosides. This characteristic elution order may be used to tentatively assign peaks based on their retention times relative to confirmed compounds.

Clear chromatographic separation was obtained between the *cis*- and *trans* isomers of the 3-O-coumaroylglucosides. The neutral loss chromatogram (extracted ion at $m/z = 639$) of the isomers of malvidin-3-O-coumaroylglucoside is shown in Fig. 2C. The *cis*-isomers of the 3-O-caffeoylglucosides [13] were not detected in any of the wines analysed here.

Two groups of anthocyanidin-diglucosides detected in this *V. vinifera* variety wine eluted before their corresponding anthocyanidin-monoglucosides, with the 3,5-diglucosides eluting before the 3,7-diglucoside isomeric forms, as described by [13]. The presence of these compounds was confirmed by the analysis of the non-*V. vinifera* variety Dornfelder, which contains higher levels of diglucosides (results not shown). Three anthocyanidin–galactosides were also observed in the studied wines; these displayed identical fragmentation patterns compared to the corresponding glucoside isomers, but eluted earlier in the RP-LC separation [23,24]. Furthermore, both dimeric anthocyanins di-malvidin-diglucoside and malvidin-glucoside-malvidin-acetyl-glucoside [25–27] were detected in neutral loss mode (observed in traces for the loss of glucosides at 162 and 324 and acetyl-glucoside at 204, respectively). Oligomeric anthocyanins have been identified in grape skins [27], red wine [13,17] and grape extracts [26]. These compounds are characterized by very broad peak shapes due to the effect of secondary equilibria involving the anthocyanins [17].

The identities of the compounds detected in neutral loss mode were further confirmed by means of the relevant aglycone cation spectrum acquired in survey scan mode. Fig. 3 shows the product ion spectra obtained for the five wine anthocyanidin-monoglucosides, whilst Table 2 lists the most prominent ions and their intensities. The fragments reported in Table 2 were confirmed by accurate mass measurements on a Q-TOF instrument (Supplementary Information, Table S1). Accurate mass measurements of the aglycone fragments all showed better than 5 ppm mass difference from the theoretical molecular formula values for the fragments ([1], Tables 2 and S1). These product ion spectra were consistent for all monoglucosides, irrespective of the modification to the sugar moieties, and were used to identify the aglycone cation for each of the anthocyanidin-monoglucosides in Table 1.

Some of the major fragmentation patterns observed for the anthocyanidins are illustrated in Fig. 4. In addition to the indicated fragmentation pathways, the product ion spectra also displayed fragments corresponding to the loss of CH_3, CH_3OH, CO and/or H atoms or radicals from the respective anthocyanidins. These fragmentation processes generally predominate over fragmentation related to rupture of the C-ring bonds (see Fig. 4 and Table 2). The product ion spectra of hydroxylated anthocyanins (cyanidin and delphinidin) are characterized by the molecular ion (aglycone cation) as the base peak, whilst the methoxylated anthocyanins (malvidin, petunidin and peonidin) also show relatively intense peaks for the molecular ion. The base peaks in the spectra for petunidin and peonidin result from the loss of a methyl group, whilst the base peak in the spectrum of malvidin results from the simultaneous elimination of a methyl group and a proton. All five wine anthocyanidins yielded the $[^{0,2}A_0]^{\bullet+}$ radical cation ($m/z = 150$) resulting from cleavage of the 0/2 C-ring bonds (Fig. 4).

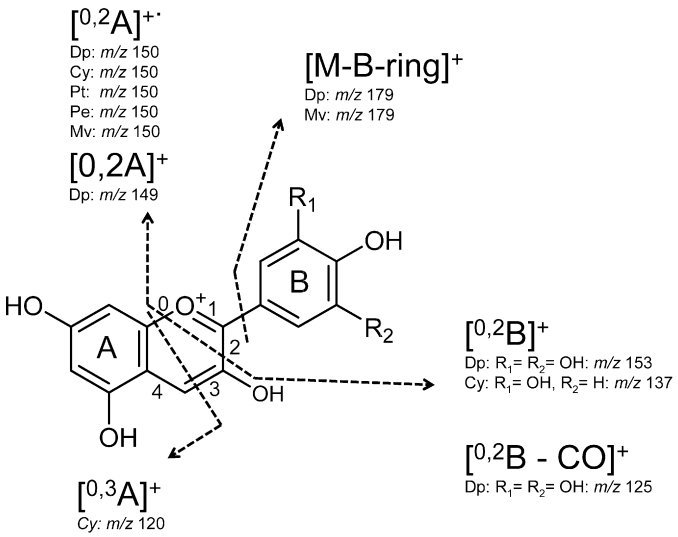

Fig. 4. Typical fragmentation patterns involving the C-ring for anthocyanidin aglycone cations under high collision energies (Table 2). Dp, delphinidin; Cy, cyanidin; Pt, petunidin; Pe, peonidin; Mv, malvidin.

The presence of this fragment in the product ion spectra may be ascribed to resonance-stabilization of the formed $[^{0,2}A_0]^{\bullet+}$ radical [1]. Only delphinidin yields the $[^{0,2}A_0]^{\bullet+}$ cation ($m/z = 149$). A similar cleavage of the 0/2 C-ring also results in the corresponding $[^{0,2}B_0]^+$ ion, yielding fragments at $m/z = 153$ and 137 for delphinidin and cyanidin, respectively. Delphinidin displays a fragment due to the further loss of CO from the $[^{0,2}B_0]^+$ ion to yield $[^{0,2}B_0{-}CO]^+$ ($m/z = 125$). Malvidin and cyanidin shows fragments at $m/z = 179$ due the loss of the B-ring, whilst cyanidin has a weak fragment corresponding to the $[^{0,3}A_0]^+$ ion at $m/z = 120$.

The anthocyanidin-diglucosides displayed aglycone cation fragmentation spectra that contained identical fragments compared to the corresponding anthocyanidin-glucosides, but present at different relative intensities. This phenomenon may be a result of the presence of the substituted sugar on the 5 and 7 positions of the A-ring, which affects fragmentation of these molecules. Fig. 5 shows the product ion spectra obtained for the two groups of malvidin-diglucosides.

3.2. Pyranoanthocyanins

Some wine constituents possessing a polarizable double bond react with the anthocyanins (monoglucosides as well as acylated derivatives) to induce cyclyzation between C-4 and the hydroxyl on C-5 of the anthocyanidin. Compounds such as pyruvic acid [9,10], acetaldehyde [11], acetone [28], 4-vinylcatechol [29], 4-vinylphenol [12], 4-vinylguaiacol [14] and vinylcatechin participate in these cycloaddition reactions [3,13,14]. In the case of 4-vinylphenol, 4-vinylguaiacol, 4-vinylcatechol and 4-vinylsyringol, adduct formation is the result of the reaction between the relevant hydroxycinnamic acids (coumaric acid, ferulic acid, caffeic acid and synaptic acid, respectively) and the anthocyanin [30]. Subsequent decarboxylation and oxidation of the intermediate carbenium ion leads to the formation of these pyranoanthocyanins [30].

Importantly, the pyranoanthocyanins are also detected selectively in neutral loss scanning mode via elimination of the relevant glycosyl moieties as described above for the anthocyanins [14]. Table 3 summarizes the chromatographic and mass spectral parameters for the pyranoanthocyanins detected in the Pinotage wines under investigation. Retention of pyranoanthocyanins in RP-LC increased with increasing

Table 2
Characteristic product ion spectra (*m/z*) obtained for the five anthocyanidin bases (% relative intensity in parenthesis).

Product ions	Delphinidin	Cyanidin	Petunidin	Peonidin	Malvidin
$[M]^+$	303(100)	287(100)	317(46)	301(27)	331(23)
$[M-CH_3]^{\bullet+}$			302(100)	286(100)	
$[M-(CH_3+H)]^+$					315(100)
$[M-CH_3OH]^+$					299(24)
$[M-(H+CH_3OH)]^{\bullet+}$				268(12)	
$[M-CH_3-CO]^{\bullet+}$			274(53)	258(62)	
$[M-CH_3-(H+CO)]^+$					287(51)
$[M-CH_3OH-(H+CO)]^{\bullet+}$					270(24)
$[M-CH_3-2CO)]^{\bullet+}$				230(29)	
$[M-CH_3-CO-(H+CO)]^+$			245(76)	229(30)	
$[M-CH_3OH-2CO]^+$				213(15)	
$[M-CH_3-3CO)]^{\bullet+}$				202(32)	
$[M-CH_3-2CO-(H+CO)]^+$			217(29)	201(38)	
$[M-CH_3OH-(H+CO)-CO]^{\bullet+}$			228(22)		242(51)
$[M-CH_3-CO-(H+CO)-C_2H_2O]^+$			203(43)		
$[M-(H+CO)]^{\bullet+}$		258(4)			
$[M-CH_3OH-2(H+CO)-CO]^+$					213(10)
$[M-H_2O-CO]^+$	257(33)	241(22)			
$[M-(H+CO)-CO]^{\bullet+}$		230(10)			
$[M-H_2O-2CO]^+$	229(48)	213(20)			
$[M-H_2O-3CO]+$	201(12)	185(5)			
$[M-B\text{-ring}]^+$	179(8)				179(18)
$[M-H_2O-4CO]^+$	173(20)				
$[^{0,2}A_0]^{\bullet+}$	150(13)	150(8)	150(9)	150(5)	150(17)
$[^{0,2}A_0]^+$	149(16)				
$[^{0,2}B_0]^+$	153(27)	137(59)			
$[^{0,3}A_0]^+$		120(3)			
$[^{0,2}B_0-CO]^+$	125(7)				

hydrophobicity of the adduct, with the following elution order: vinylformic acid < acetaldehyde < acetone < 4-vinylcatechol < 4-vinylphenol < 4-vinylguaiacol. Within each class, the elution order was the same as observed for the unmodified anthocyanins.

Significantly, the mass spectra of the aglycone cations of pyranoanthocyanins obtained in survey scan mode are characterized by the elimination of identical fragments compared to the corresponding unmodified anthocyanins [14]. This is due to the fact that modification to the anthocyanidin base occurs between C-4 and C-5. Since the primary fragmentation of anthocyanidins involves cleavage of the bonds between C-1 and C-4 (Fig. 4), identical losses are also observed for the pyranoanthocyanins (the exception being for the $[^{0,3}A]^+$ fragment of cyanidin-glucoside). This observation also confirms that the loss of loss of CH_3, CH_3OH, CO and H fragments occurs at the B-ring of the anthocyanidins.

This fragmentation behaviour means that for the pyranoanthocyanidins the fragment ions obtained are offset by a consistent mass compared to the corresponding parent anthocyanidin, with the offset determined by the mass of the relevant adduct. For example, the fragments in the product ion spectra of the vinylformic acid adducts are offset by 68 amu compared to the characteristic fragment ions of the unmodified anthocyanidin base. Similarly, for the acetaldehyde, acetone, 4-vinylcatechol, 4-vinylphenol and 4-vinylguaiacol derivatives this offset corresponds to 24, 38, 132, 116

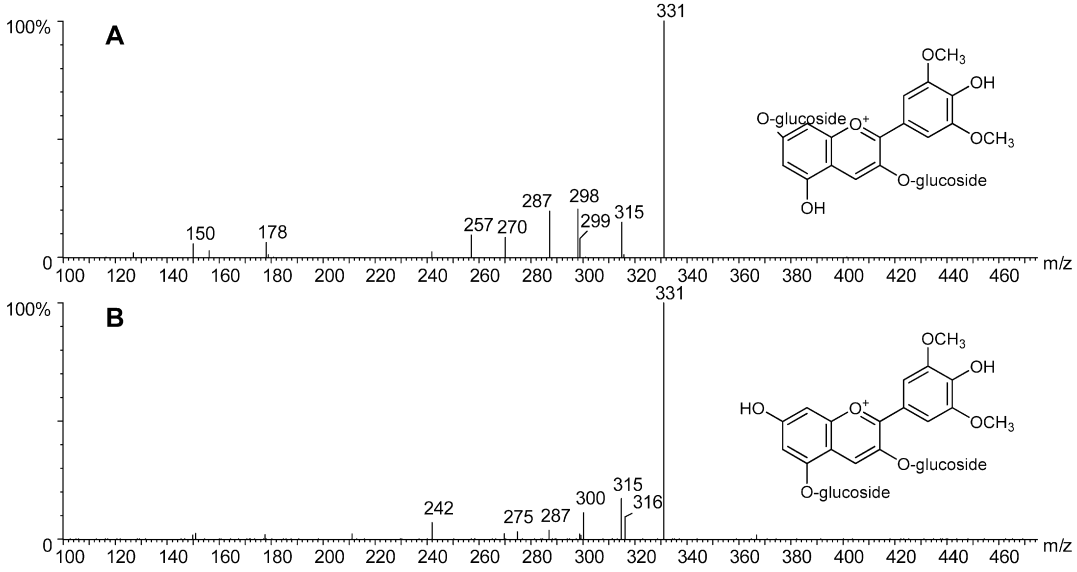

Fig. 5. Characteristic product ion mass spectra as well as structures of (A) malvidin-3,7-di-O-glucoside (peak 45, Table 1) and (B) malvidin-3,5-di-O-glucoside (peak 30, Table 1). Note the differing intensities of the ions compared to Fig. 3(E) for malvidin-3-O-glucoside.

Table 3
Chromatographic and mass spectrometric parameters of detected pyranoanthocyanins and anthocyanin-flavonol condensation products.

Peak no[a]	Compound[b]	Retention time (min)	M^+ (m/z)	Neutral loss offset (amu)
15	Vinylformic acid adduct of Dp-3-O-glucoside	9.74[c]	533	162
22	Vinylformic acid adduct of Cy-3-O-glucoside	12.00	517	162
28	Vinylformic acid adduct of Pt-3-O-glucoside	13.10	547	162
46	Vinylformic acid adduct of Pe-3-O-glucoside	15.17[c]	531	162
47	Vinylformic acid adduct of Mv-3-O-glucoside	15.99	561	162
17	Vinylformic acid adduct of Dp-3-O-acetylglucoside	11.04[c]	575	204
32	Vinylformic acid adduct of Cy-3-O-acetylglucoside	13.75[c]	559	204
38	Vinylformic acid adduct of Pt-3-O-acetylglucoside	14.33[c]	589	204
54	Vinylformic acid adduct of Pe-3-O-acetylglucoside	17.07[c]	573	204
57	Vinylformic acid adduct of Mv-3-O-acetylglucoside	17.46	603	204
44	Vinylformic acid adduct of Dp-3-O-coumaroylglucoside	15.12[c]	679	308
51	Vinylformic acid adduct of Cy-3-O-coumaroylglucoside	16.90[c]	663	308
58	Vinylformic acid adduct of Pt-3-O-coumaroylglucoside	17.80[c]	693	308
74	Vinylformic acid adduct of Pe-3-O-coumaroylglucoside	20.12[c]	677	308
77	Vinylformic acid adduct of Mv-3-O-coumaroylglucoside	20.34	707	308
43	Vinylformic acid adduct of Mv-3-O-caffeoylglucoside	15.11	723	324
19	Acetaldehyde adduct of Dp-3-O-glucoside	11.11	489	162
27	Acetaldehyde adduct of Cy-3-O-glucoside	13.04	473	162
36	Acetaldehyde adduct of Pt-3-O-glucoside	14.29	503	162
50	Acetaldehyde adduct of Pe-3-O-glucoside	16.17	487	162
53	Acetaldehyde adduct of Mv-3-O-glucoside	17.03	517	162
29	Acetaldehyde adduct of Dp-3-O-acetylglucoside	13.13[c]	531	204
48	Acetaldehyde adduct of Pt-3-O-acetylglucoside	16.09[c]	545	204
64	Acetaldehyde adduct of Pe-3-O-acetylglucoside	18.40[c]	529	204
67	Acetaldehyde adduct of Mv-3-O-acetylglucoside	18.85	559	204
59	Acetaldehyde adduct of Dp-3-O-coumaroylglucoside	17.81[c]	635	308
89	Acetaldehyde adduct of Pe-3-O-coumaroylglucoside	21.45[c]	633	308
91	Acetaldehyde adduct of Mv-3-O-coumaroylglucoside	21.66[c]	663	308
37	Acetone adduct of Dp-3-O-glucoside	14.29	503	162
49	Acetone adduct of Cy-3-O-glucoside	16.13[c]	487	162
55	Acetone adduct of Pt-3-O-glucoside	17.08[c]	517	162
65	Acetone adduct of Pe-3-O-glucoside	18.50[c]	501	162
70	Acetone adduct of Mv-3-O-glucoside	19.18[c]	531	162
85	Acetone adduct of Mv-3-O-acetylglucoside	20.70	573	204
73	Acetone adduct of Dp-3-O-coumaroylglucoside	20.03[c]	649	308
102	Acetone adduct of Pe-3-O-coumaroylglucoside	22.90[c]	647	308
103	Acetone adduct of Mv-3-O-coumaroylglucoside	23.17[c]	677	308
62	4-Vinylcatechol adduct of Dp-3-O-glucoside	18.19	597	162
82	4-Vinylcatechol adduct of Pt-3-O-glucoside	20.61	611	162
96	4-Vinylcatechol adduct of Pe-3-O-glucoside	22.19[c]	595	162
99	4-Vinylcatechol adduct of Mv-3-O-glucoside	22.51	625	162
106	4-Vinylcatechol adduct of Pe-3-O-acetylglucoside	23.45[c]	637	204
107	4-Vinylcatechol adduct of Mv-3-O-acetylglucoside	23.55[c]	667	204
101	4-Vinylcatechol adduct of Pt-3-O-coumaroylglucoside	22.88[c]	757	308
111	4-Vinylcatechol adduct of Pe-3-O-coumaroylglucoside	24.58[c]	741	308
112	4-Vinylcatechol adduct of Mv-3-O-coumaroylglucoside	24.74[c]	771	308
72	4-Vinylphenol adduct of Dp-3-O-glucoside	19.84[c]	581	162
78	4-Vinylphenol adduct of Cy-3-O-glucoside	20.34[c]	565	162
94	4-Vinylphenol adduct of Pt-3-O-glucoside	21.99[c]	595	162
104	4-Vinylphenol adduct of Pe-3-O-glucoside	23.35[c]	579	162
108	4-Vinylphenol adduct of Mv-3-O-glucoside	23.68	609	162
88	4-Vinylphenol adduct of Dp-3-O-acetylglucoside	20.95[c]	623	204
105	4-Vinylphenol adduct of Pt-3-O-acetylglucoside	23.41[c]	637	204
113	4-Vinylphenol adduct of Pe-3-O-acetylglucoside	24.75	621	204
114	4-Vinylphenol adduct of Mv-3-O-acetylglucoside	24.98	651	204
118	4-Vinylphenol adduct of Pe-3-O-coumaroylglucoside	26.19[c]	725	308
119	4-Vinylphenol adduct of Mv-3-O-coumaroylglucoside	26.30	755	308
84	4-Vinylguaiacol adduct of Dp-3-O-glucoside	20.66[c]	611	162
95	4-Vinylguaiacol adduct of Cy-3-O-glucoside	22.16[c]	595	162
100	4-Vinylguaiacol adduct of Pt-3-O-glucoside	22.66[c]	625	162
109	4-Vinylguaiacol adduct of Pe-3-O-glucoside	23.89[c]	609	162
110	4-Vinylguaiacol adduct of Mv-3-O-glucoside	24.29	639	162
93	4-Vinylguaiacol adduct of Dp-3-O-acetylglucoside	21.83[c]	653	204
116	4-Vinylguaiacol adduct of Pe-3-O-acetylglucoside	25.33[c]	651	204
117	4-Vinylguaiacol adduct of Mv-3-O-acetylglucoside	25.59[c]	681	204
115	4-Vinylguaiacol adduct of Pt-3-O-coumaroylglucoside	25.04[c]	771	308
120	4-Vinylguaiacol adduct of Pe-3-O-coumaroylglucoside	26.43[c]	755	308
121	4-Vinylguaiacol adduct of Mv-3-O-coumaroylglucoside	26.83[c]	785	308
6	Catechin adduct of Dp-3-O-glucoside (T-A)	5.40[c]	753	162
8	Catechin adduct of Pt-3-O-glucoside (T-A)	6.96[c]	767	162
11	Catechin adduct of Pe-3-O-glucoside (T-A)	8.77[c]	751	162
14	Catechin adduct of Mv-3-O-glucoside (T-A)	9.43[c]	781	162
31	Catechin adduct of Pe-3-O-acetylglucoside (T-A)	13.69[c]	793	204
40	Catechin adduct of Mv-3-O-acetylglucoside (T-A)	14.58[c]	823	204
63	Catechin adduct of Mv-3-O-coumaroylglucoside (T-A)	18.26[c]	927	308

Table 3 (*Continued*)

Peak no[a]	Compound[b]	Retention time (min)	M^+ (*m*/*z*)	Neutral loss offset (amu)
20	Epi-catechin adduct of Mv-3-O-glucoside (T-A)	11.50[c]	781	162
1	Gallocatechin adduct of Pt-3-O-glucoside (T-A)	3.05[c]	783	162
2	Gallocatechin adduct of Pe-3-O-glucoside (T-A)	3.51[c]	767	162
3	Gallocatechin adduct of Mv-3-O-glucoside (T-A)	4.11[c]	797	162
4	Epi-gallocatechin adduct of Pt-3-O-glucoside (T-A)	4.58[c]	783	162
5	Epi-gallocatechin adduct of Pe-3-O-glucoside (T-A)	5.19[c]	767	162
7	Epi-gallocatechin adduct of Mv-3-O-glucoside (T-A)	5.79[c]	797	162

[a] Peak numbers correspond to Fig. 2.
[b] Dp, delphinidin; Cy, cyanidin; Pt, petunidin; Pe, peonidin; Mv, malvidin.
[c] Survey scan spectra do not allow conclusive identification of aglycone due to low signal intensity.

and 146 amu, respectively. The pyranoanthocyanins may therefore also be identified by applying the criteria outlined above for the anthocyanins. Fig. 6 shows the characteristic aglycone cation mass spectra obtained for malvidin-3-O-glucoside congeners of selected pyranoanthocyanins. It is important to note, however, that the relative intensities of some of the characteristic fragments are different compared to malvidin-3-O-glucoside (Fig. 3(E)). The vinylformic acid-, acetaldehyde-, acetone-, 4-vinylcatechol-, 4-vinylphenol- and 4-vinylguaiacol-adducts of anthocyanins and their acylated derivatives were identified (Table 3).

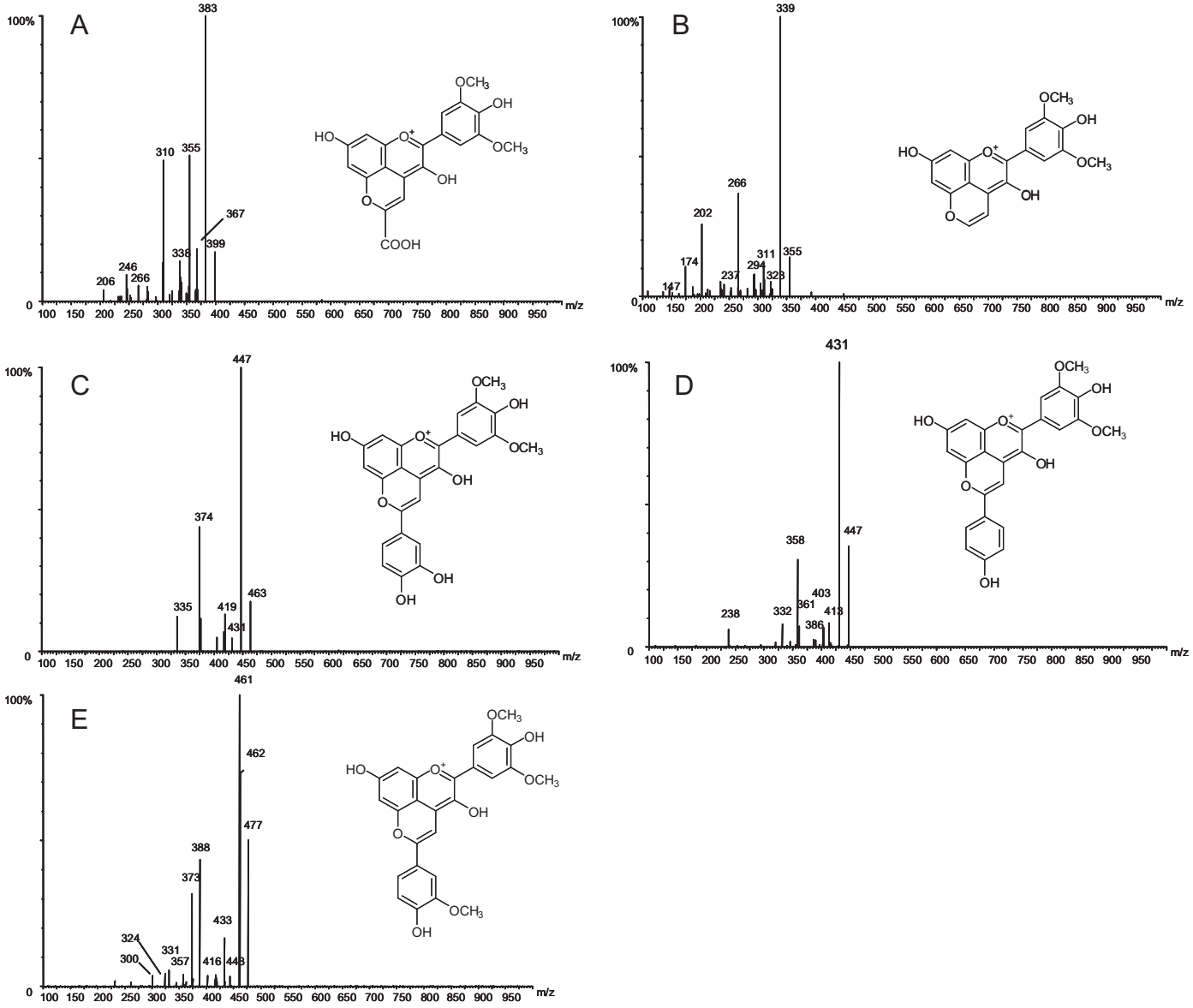

Fig. 6. Characteristic survey scan product ion mass spectra and structures of the pyranoanthocyanin derivatives of malvidin-3-O-glucoside, (A) vinylformic acid adduct, (B) acetaldehyde adduct, (C) vinylcatechol adduct, (D) vinylphenol adduct and (E) vinylguaiacol adduct.

3.3. Direct and acetaldehyde-mediated anthocyanin-flavanol condensation products

Several proanthocyanidin-anthocyanin adducts were also detected in neutral loss scanning mode. These compounds are formed by reaction of procyanidins and anthocyanins via coupling between C-4 of the anthocyanidin and either C-6 or C-8 of an (epi)catechin moiety of the procyanidin molecule (referred to as A-T condensation products) or between C-6 or C-8 of the anthocyanidin and C-6 or C-8 of the procyanidin (T-A condensation) [4,6,31]. The latter compounds occur in the bicyclic form [31,32] and elute before the corresponding A-T products under RP-LC conditions [17]. Only the T-A type anthocyanin-flavanol adducts containing one catechin or epicatechin unit were detected in the Pinotage wines investigated here (Table 3). The fact that no A-T condensation products or higher molecular weight T-A adducts were detected might be because of additional fragmentation patterns involving the procyanidin moiety, such as loss of (epi)catechin (m/z 288) and retro-Diels-Alder fission of the (epi)catechin units (m/z 152) [17]. In fact, additional experiments were performed in neutral loss mode by monitoring these losses, but these only allowed detection of free procyanidins in the wines studied. However, since especially the higher molecular weight anthocyanin-procyanidin adducts are present in relatively low concentrations, the additional fragmentation pathways involving these molecules (which are expected to become predominant with increase in the size of the procyanidin group) are presumably responsible for the fact that none of these compounds were detected in neutral loss mode utilizing the characteristic losses for anthocyanins only.

Six prodelphinidin-anthocyanin adducts were also detected in neutral loss scanning mode. These compounds eluted before the corresponding procyanidin-anthocyanin adducts [17]. The peak shapes for both prodelphinidin- and procyanidin-anthocyanin adducts were relatively broad due to the effect of secondary equilibria involving the anthocyanin moiety of these compounds [17].

It is also worth noting that no vinylcatechin adducts of anthocyanins, where the anthocyanin is linked at the C-4 position via a vinyl linkage to a procyanidin, were detected in the current study [33]. This may once again be due to the presence of additional fragmentation patterns of these molecules, which prohibit their detection at low levels using the neutral loss experiments performed here.

Note that the approach used in the current contribution was developed specifically to exploit the benefits of triple quadrupole instrumentation. Alternative mass analysers can also be used advantageously for anthocyanin analysis. For example, high resolution TOF instruments provide the benefits of accurate mass for identification purposes, whereas Q-TOF instruments provide improved sensitivity in full scan mode and can be used to obtain accurate mass spectra of the daughter anthocyanidins. However, Q-TOF instruments cannot be operated in neutral loss mode, and are therefore not suited for the selective screening of wine anthocyanins in a similar manner as used in the current contribution. Nor can neutral-loss-directed survey scans be performed on such instrumentation. Therefore, whilst alternative mass analysers can beneficially be applied for anthocyanin analysis, a similar selective screening and identification approach as reported here requires the use of triple quadrupole (or ion-trap) instrumentation. Considering the relatively lower cost and more widespread availability of triple quadrupole instruments, the method reported here shows promise for the detailed screening of pigmented fractions of wine.

4. Conclusions

A systematic approach for the detailed investigation of red wine pigments has been developed by exploiting the advantages inherent to optimized chromatographic separation in combination with the selective detection capabilities and structural identification power of tandem mass spectrometry utilizing a triple quadrupole instrument. The proposed approach involves two sets analyses: in the first instance, neutral loss scanning is performed for losses of 162, 204, 308 and 324 amu to selectively detect anthocyanin glucosides, diglucosides and acylated derivatives, as well as the corresponding derivatives formed during wine ageing. This is followed by 4 survey scan experiments to identify the aglycone cation based on characteristic fragmentation processes for the anthocyanidins. The highly selective nature of neutral loss detection simplifies the analysis, and in combination with the structured RP-LC chromatographic elution order allows tentative compound identification. Moreover, neutral loss scanning reveals the molecular weight and mass of the attached sugar moiety, which further aids compound identification. Characterization of the aglycone cation by a second set of experiments in survey scan mode ensures unambiguous identification of detected compounds. The highly selective nature of the neutral loss experiments also makes this mode of detection inherently suited to quantitative determination of these compounds. The limits of detection under our conditions are approximately 0.1 mg/L in neutral loss mode, whilst consistent product ion mass spectra were obtained in survey scan mode at concentrations above 5 mg/L (as determined for malvidin-3-O-glucoside). This methodology therefore offers a powerful and simple approach for the selective determination of the anthocyanins and derived compounds in red wines. This method allowed us to identify a total of 121 different compounds belonging to the anthocyanins, pyranoanthocyanins, and flavanol-anthocyanin condensation products in Pinotage wines. The primary benefits of this approach are two-fold: increased selectivity simplifies identification and minimizes co-elution so that the structured RP-LC elution may be exploited to allow more accurate tentative compound identification. Secondly, product ion spectra significantly increase the certainty in identification (at least of major compounds). The MS/MS spectra reported here for the first time for anthocyanin derivatives in wine will prove useful in minimizing the risks of false identification, which is always a factor due to the unavailability of standards for wine anthocyanins. The method therefore offers a simple and fast approach for qualitative screening of red wine anthocyanins where the use of RP-LC overcome some of the limitations of direct infusion mass spectrometry such as ion suppression as well as wrong assignment of compounds due to identical masses of many wine pigments. Further research is required to establish the suitability of the proposed methodology for quantitative analysis. This would allow more accurate and detailed investigation of the evolution of wine anthocyanins and their derived products during wine ageing.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.02.058.

References

[1] B. Abad-García, L.A. Berrueta, S. Garmón-Lobato, B. Gallo, F. Vicente, J. Chromatogr. A 1216 (2009) 5398.
[2] B. Berente, D.D. García, M. Reichenbächer, K. Danzer, J. Chromatogr. A 871 (2000) 95.
[3] R.S. Jackson, Wine Science: Principles, Practice and Perception, 2nd ed., Academic Press, San Diego, U.S.A., 2000.
[4] B.W. Zoecklein, K.C. Fugelsang, B.H. Gump, F.S. Nury, Production Wine Analysis, Chapman & Hall, New York, U.S.A., 1990.
[5] E.M. Francia-Aricha, M.T. Guarra, J.C. Rivas-Gonzalo, C. Santos-Buelga, J. Agric. Food Chem. 45 (1997) 2262.
[6] S. Remy, H. Fulcrand, B. Labarbe, V. Cheynier, M. Moutounet, J. Sci. Food Agric. 80 (2000) 745.

[7] J.C. Rivas-Gonzalo, S. Bravo-Haro, C. Santos-Buelga, J. Agric. Food Chem. 43 (1995) 1444.
[8] E. Salas, V. Atanasova, C. Poncet-Legrand, E. Meudec, J.P. Mazauric, V. Cheynier, Anal. Chim. Acta 513 (2004) 325.
[9] H. Fulcrand, C. Benabdeljalil, J. Rigaud, V. Cheynier, M. Moutounet, Phytochemistry 47 (1998) 1401.
[10] C. Romero, J. Bakker, J. Agric. Food Chem. 47 (1999) 3130.
[11] J. Bakker, C.F. Timberlake, J. Agric. Food Chem. 45 (1997) 35.
[12] H. Fulcrand, P.J. Cameira dos Santos, P. Sarni-Manchado, V. Cheynier, J. Favre-Bonvin, J. Chem. Soc., Perkin Trans. 1 (1996) 735.
[13] C. Alcalde-Eon, M.T. Escribano-Bailón, C. Santos-Buelga, J.C. Rivas-Gonzalo, Anal. Chim. Acta 563 (2006) 238.
[14] Y. Hayasaka, R.E. Asenstrofer, J. Agric. Food Chem. 50 (2002) 756.
[15] N. Mateus, A.M. Silva, J.C. Rivas-Gonzalo, C. Santos-Buelga, V. De Freitas, J. Agric. Food Chem. 51 (2003) 1919.
[16] A. de Villiers, D. Cabooter, F. Lynen, G. Desmet, P. Sandra, J. Chromatogr. A 1216 (2009) 3270.
[17] A. de Villiers, D. Cabooter, F. Lynen, G. Desmet, P. Sandra, J. Chromatogr. A 1218 (2011) 4660.
[18] D. Favretto, R. Flamini, Am. J. Enol. Vitic. 51 (2000) 55.
[19] V. Atanasova, H. Fulcrand, V. Cheynier, M. Moutounet, Anal. Chim. Acta 458 (2002) 15.
[20] A. de Villiers, G. Vanhoenacker, P. Majek, P. Sandra, J. Chromatogr. A 1054 (2004) 195.
[21] J.Y. Choi, S.J. Lee, S.J. Lee, S. Park, J.H. Lee, J.H. Shim, A.M.A. El-Aty, J.S. Jin, E.D. Jeong, W.S. Lee, S.C. Shin, J. Sep. Sci. 33 (2010) 1192.
[22] K. Nagy, K. Redeuil, R. Bertholet, H. Steiling, M. Kussmann, Anal. Chem. 81 (2009) 6347.
[23] L.G. Wade, Organic Chemistry, 4th ed., Prentice-Hall Inc., Upper Saddle River, New Jersey, U.S.A., 1999.
[24] H. Wang, E.J. Race, A.J. Shrikhande, J. Agric. Food Chem. 51 (2003) 1839.
[25] C. Alcalde-Eon, M.T. Escribano-Bailón, C. Santos-Buelga, J.C. Rivas-Gonzalo, J. Mass Spectrom. 42 (2007) 735.
[26] S. Pati, I. Losito, G. Gambacorta, E.L. Notte, F. Palmisano, P.G. Zambonin, J. Mass Spectrom. 41 (2006) 861.
[27] S. Vidal, E. Meudec, V. Cheynier, G. Skouroumounis, Y. Hayasaka, J. Agric. Food Chem. 52 (2004) 7144.
[28] Y. Lu, L.Y. Foo, Tetrahedron Lett. 42 (2001) 1371.
[29] A.E. Håkansson, K. Pardon, Y. Hayasaka, M. De Sa, M. Herderich, Tetrahedron Lett. 44 (2003) 4887.
[30] M. Schwarz, T.C. Wabnitz, P. Winterhalter, J. Agric. Food Chem. 51 (2003) 3682.
[31] M. Monagas, B. Bartolomé, C. Gómez-Cordovés, Food Sci. Nutr. 45 (2005) 85.
[32] S. Remy-Tanneau, C. Le Guerneve, E. Meudec, V. Cheynier, J. Agric. Food Chem. 51 (2003) 3592.
[33] J. He, C. Santos-Buelga, N. Mateus, V. de Freitas, J. Chromatogr. A 1134 (2006) 215.