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Determination of anthocyanins in wine by direct injection liquid chromatography–diode array detection–mass spectrometry and classification of wines using discriminant analysis

A. de Villiers[a], G. Vanhoenacker[b], P. Majek[c], P. Sandra[a,b,*]

[a] Department of Chemistry, University of Stellenbosch, CENSSUS, Private Bag X1, Matieland 7602, South Africa
[b] Research Institute for Chromatography, University of Gent, Kennedypark 20, B-8500 Kortrijk, Belgium
[c] Department of Analytical Chemistry, Slovak University of Technology, 81237 Bratislava 1, Slovak Republic

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Abstract

A rapid HPLC–diode array detection (DAD) method was developed for the routine analysis of 16 anthocyanins in wine. Direct injection of filtered wine samples followed by selective detection at 520 nm allowed quantitation of these compounds in red wines. The method was linear for malvidin-3-glucoside over the range 5–250 ppm, and the limit of detection for this compound was 0.18 ppm. A volatile mobile phase is used, which enables hyphenation to mass spectrometry (MS). With HPLC–MS, a total of 44 pigments could be identified in South African wines. Obtained mass spectra are discussed for a series of representative wine constituents and results are compared with literature references. An attempt was made to differentiate between different cultivars according to the anthocyanin content using stepwise forward linear discriminant analysis (LDA).

Keywords: Anthocyanins; Wine

1. Introduction

Anthocyanins (anthocyanidin-glycosides) are naturally occurring pigments responsible for the colour of many fruits, including grapes, vegetables and flowers. These flavonoids are characterised by the cationic flavylium structure (Table 1), which is predominant only at low pH. In wine media, bleaching by bisulphite and oxidation reactions also take place, ensuring that only a relatively small percentage of the anthocyanins is present in their red flavylium cationic form [1].

The anthocyanins are extracted from the skins of black grapes during maceration, becoming responsible for the purple-red colour of young wines. During ageing, however, the levels of grape anthocyanins rapidly decrease as they react with a variety of other wine constituents [1]. This process, leading to the formation of more stable pigments, is responsible for the change in colour (from purple-red to brick-red) as well as the loss of astringency observed during wine ageing [2]. A number of pathways for these conversions have been proposed and demonstrated. Condensation of anthocyanins with flavanols, either directly [3,4], or mediated by acetaldehyde [5], has been shown to occur. Cyclo-addition reactions at C4 involving vinyl-phenol derivatives [6–8], pyruvic acid [9–12], acetaldehyde [10] and procyanidin B2 [13] have been reported. These derived pigments are more resistant to increase in pH and bisulphite bleaching, and are orange-red [10,12]. Furthermore, non-covalent interaction between anthocyanins and other phenolics, known as co-pigmentation, influences the colour of the young red wine, and might be the first step in the formation of pigmented condensed tannins [3].

All reactions mentioned above contribute to the colour and colour stability of wine, and can influence the organoleptic properties through their effect on the wine tannin structure. In fact, correlation between wine quality ratings and colour densities (primarily determined by the degree of ionisation of anthocyanins) has been demonstrated for young Australian

* Corresponding author. Tel.: +32 56 204 031; fax: +32 56 204 859.
E-mail address: pat.sandra@richrom.com (P. Sandra).

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Table 1
Structures of the pigments identified in wine

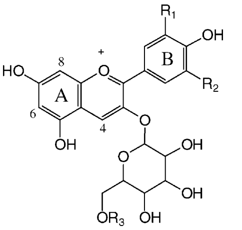
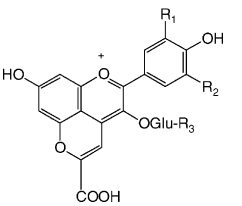
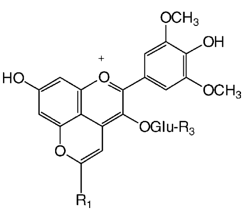
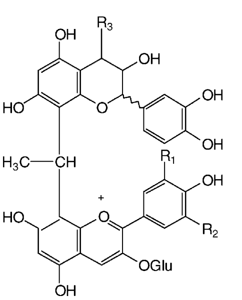
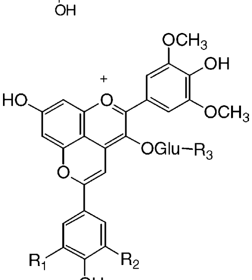
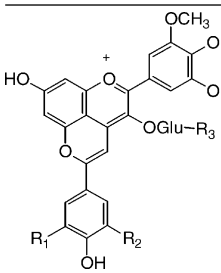
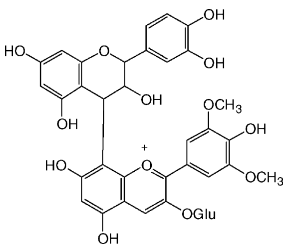
Structure	Name	R1	R2	R3
	Dp-3-glucoside (**1**)	OH	OH	H
	Cy-3-glucoside (**2**)	OH	H	H
	Pt-3-glucoside (**3**)	OCH_3	OH	H
	Pe-3-glucoside (**4**)	OCH_3	H	H
	Mv-3-glucoside (**5**)	OCH_3	OCH_3	H
	Dp-acetyl-glucoside (**6**)	OH	OH	Acetyl
	Cy-acetyl-glucoside (**7**)	OH	H	Acetyl
	Pt-acetyl-glucoside (**8**)	OCH_3	OH	Acetyl
	Pe-acetyl-glucoside (**9**)	OCH_3	H	Acetyl
	Mv-acetyl-glucoside (**10**)	OCH_3	OCH_3	Acetyl
	Dp-coumaroyl-gluc (**11**)	OH	OH	Coumaroyl
	Cy-coumaroyl-gluc (**12**)	OH	H	Coumaroyl
	Pt-coumaroyl-gluc (**13**)	OCH_3	OH	Coumaroyl
	Pe-coumaroyl-gluc (**14**)	OCH_3	H	Coumaroyl
	Mv-coumaroyl-gluc (**15**)	OCH_3	OCH_3	Coumaroyl
	Mv-caffeoyl-gluc (**28**)	OCH_3	OCH_3	Caffeoyl
	Pt-3-glucoside-pyruvic acid[a] (**17**)	OCH_3	OH	H
	Pe-3-glucoside-pyruvic acid[a] (**18**)	OCH_3	H	H
	Vitisin A[a] (**19**)	OCH_3	OCH_3	H
	Acetylvitisin A[a] (**23**)	OCH_3	OCH_3	Acetyl
	Coumaroylvitisin A (**27**)	OCH_3	OCH_3	Coumaroyl
	Vitisin B (**20**)	H		H
	Acetylvitisin B (**25**)	H		Acetyl
	Coumaroylvitisin B (**29**)	H		Coumaroyl
	Mv-glucoside-vinyl-(epi)catechin (**30**, **33**)	Cat/ecat		H
	Mv-glucoside-ethyl-(epi)catechin (**21**, **24**, **26**)	OCH_3	OCH_3	H
	Mv-glucoside-ethyl-(epi)catechin-unknown (**22**)	OCH_3	OCH_3	Unknown
	Pigment A (**36**)	H	H	H
	Acetyl-pigment A (**40**)	H	H	Acetyl
	Pigment B (**42**)	H	H	Coumaroyl
	Pinotin A (**32**)	H	OH	H
	Acetyl-pinotin A (**35**)	H	OH	Acetyl
	Mv-3-glucoside-vinylguaiacol (**37**)	H	OCH_3	H
	Coumaroyl-pinotin A (**39**)	H	OH	Coumaroyl
	Mv-acetyl-glucoside-vinylguaiacol (**41**)	H	OCH_3	Acetyl
	Mv-coumaroyl-gluc-vinylguaiacol (**43**)	H	OCH_3	Coumaroyl
	Mv-3-glucoside-vinylsyringol (**44**)	OCH_3	OCH_3	H

Table 1 (*Continued*)

Structure	Name	R1	R2	R3
	Pt-glucoside-4-vinylphenol (**31**)	H	H	H
	Pt-acetyl-glucoside-4-vinylphenol (**34**)	H	H	Acetyl
	Pt-coumaroyl-gluc-4-vinylphenol (**38**)	H	H	Coumaroyl
	Mv-glucoside-(epi)catechin (**16**)			

Key: dp: delphinidin; cy: cyanidin; pt: petunidin; pe: peonidin; mv: malvidin; gluc: glucose; cat/ecat: catechin/epicatechin.
[a] Structure as proposed by Fulcrand et al. [11].

and French wines [14]. Clearly, the determination of these compounds is an essential part of oenology. Not only can the replacement of the anthocyanins by the more stable derived pigments as the primary colour contributors be studied, this process can also be related to oenological practice [15], leading to new insights into the maturation process. In addition, there has been increasing interest in anthocyanins due to their antioxidant capabilities and biological activity [16]. Recently, much attention is devoted to the classification of wines based on the chemical composition. In this regard, wine anthocyanin profiles have been shown to be characteristic for each variety [17]. Indeed, in combination with chemometric methods, the differentiation of wines according to variety by anthocyanin and phenolic content has been reported [18]. Further, differentiation of German wines [19] and classification of Spanish wines [20] according to cultivar, as well as classification of wines according to geographical origin [21] has been demonstrated using anthocyanin fingerprints. Supervised pattern recognition methods are often used to derive a classification rule from a set of wines of known class, and this in turn is used to classify unknown wine samples. Specifically, the efficacy of discriminant analysis (DA) has been demonstrated by numerous authors [22,23].

Since the report of Wulf and Nagel [24], HPLC has replaced the previously used two-dimensional TLC as separation method for the determination of wine anthocyanins. Although spectral procedures according to the method of Somers and Evans [25] can be used to estimate the total anthocyanins, polymeric anthocyanins, etc., this does not allow quantification of individual compounds, and moreover leads to overestimation of free anthocyanins [26]. Capillary electrophoresis (CE) has been applied for anthocyanin analysis [27], but its applicability for wine analysis has not yet been demonstrated. Thus, HPLC in combination with UV detection has become the analysis method of choice for the determination of anthocyanins in grapes [28], fruit juices [29] and wine [6,9,12,15,26,30–32].

With the advent of reliable ionisation sources for coupling liquid chromatography (LC) to mass spectrometry (MS), LC–MS has been applied for the identification of diverse anthocyanins and derived products in fruit [33], grapes [34,35] and wines [36,37]. Characterisation of anthocyanins has also been achieved by direct infusion into the MS [38] and using MALDI–MS [39]. Despite the attractive features of these methodologies, LC–MS instrumentation is expensive and not commonly available in South African wine laboratories. Diode array detection (DAD) in combination with an enrichment technique like solid-phase extraction (SPE) or liquid–liquid extraction has been used to elucidate the main anthocyanins through their UV spectra [40]. The anthocyanin elution pattern for young wines from a reversed-phase column are characteristic enough to allow identification of the main compounds but, partly due to the lack of available standards, compounds present in trace amounts cannot be elucidated and should be identified by on-line MS detection.

The aim of the present study was in the first instance to develop an LC–ESI–MS method for the identification of anthocyanin-derived pigments in red wines. Based on the obtained results, a LC–DAD method for routine analysis of the major wine pigments without sample preparation is proposed. The application of this method to various South African red wines is briefly discussed. Data generated using this method were used in an attempt to classify South African red wine according to variety based on their anthocyanin content, using DA.

2. Experimental

2.1. Materials

HPLC grade acetonitrile and water were from Sigma–Aldrich (Atlasville, South Africa), formic acid (100%) from Acros (Geel, Belgium). Malvidin-3-glucoside chloride (Oenin chloride) was obtained from Extrasynthese (Genay, France), and dissolved in 1/19/80 HCl/water/methanol. Delphinidin and cyanidin-3-glucoside were kindly donated by the Laboratory of Pharmacognosy and Phytochemistry (Ghent University, Faculty of Pharmaceutical Sciences, Belgium). LC mobile phases and wine samples were filtered through 0.45 μm HV filters before use (Millipore Corporation, Bedford, MA, USA). Fifty-five red wines of various vintages ranging from 1988 to 2003 were purchased from local stores. Five cultivars were analysed: Cabernet Sauvignon, Merlot, Shiraz (Syrah), Pinotage and Ruby Cabernet. When not analysed from freshly opened bottled, wine samples were transferred under nitrogen to completely filled amber bottles to ensure their preservation.

2.2. Instrumentation

Method development was carried out using UV detection on an Alliance 2690 Separations Module equipped with a 996 Photodiode Array Detector (Waters, Milford, MA, USA). Data analysis was done with Millenium[32] Chromatography Manager software. A 25 cm × 4.6 mm i.d., 5 μm particles Luna C18 column (Phenomenex, Torrance, CA, USA) was used with a mobile phase consisting of (A) 7.5% formic acid, and (B) 7.5% formic acid in acetonitrile. The following optimised gradient was applied: 3% B for 1 min, 3–15% B in 11 min, 15–25% B in 12 min, 25–30% B in 4 min, and 30% B for 4 min before returning to the initial conditions. Twenty microlitres was injected and the column was thermostatted at 25 °C. The flow-rate was 1 mL min^{-1} and detection was performed at 520 nm. UV spectra over the range 200–600 nm were recorded.

LC–MS analyses were performed on a LCQ ion trap mass spectrometer (Thermo Finnigan, San José, CA, USA) equipped with an electrospray interface. A model 325 HPLC pump and UVKON model 735LC single wavelength UV detector set to 520 nm (both from KONTRON Instruments, Watford, UK) were used, together with a Uniflows DG-1310 degasser (Uniflows, Tokyo, Japan). A 25 cm × 2 mm i.d., 3 μm particles Luna C18 column (Phenomenex, Torrance, CA, USA) was used, with the same mobile phase and gradient conditions as for the LC–UV analyses and at a flow-rate of 0.4 mL min^{-1}. Positive electrospray conditions were optimised by infusion of a solution of delphinidin in phase B, and were as follows: source voltage was 3.8 kV, capillary temperature 225 °C, sheath gas and auxiliary gas (both nitrogen) 60 and 20 arbitrary units, respectively. Full scan spectra were recorded over the range *m*/*z* 100–1500. For MS–MS experiments, the molecular ion was isolated in the ion trap, followed by collision-induced dissociation (CID) at 1.5 V.

2.3. Statistical methods

A data matrix was constructed from the anthocyanin data with rows represented by wine samples (objects) and columns corresponding to anthocyanin concentrations (variables). Autoscaling was performed to produce variables with zero means and unit standard deviation [41]. Initially, univariate characterization was carried out based on Fischer's weight (*F*) by means of one-way ANOVA to establish which compounds differ significantly between varieties. Consequently, stepwise forward linear discriminant analysis (LDA) was used to derive a classification rule whereby the wine samples were classified according to variety. All statistical data analysis was performed using STATISTICA, version 6.1 (Statsoft Inc., OK, USA).

3. Results and discussion

3.1. LC–UV method development

The method was developed with the following aims in mind. Firstly, to be amenable to MS detection as identification is to be based on mass spectral data; secondly, to keep sample preparation to a minimum in order to eliminate loss of the labile anthocyanins; and finally, the method had to be rugged enough to allow UV quantitation of the compounds identified by MS.

In the first step, the mobile phase composition was optimised. The generic HPLC method for the analysis of anthocyanins is based on reversed-phase LC with gradient elution employing acidified eluents. The low pH of the mobile phase is required to ensure that the anthocyanins are in the flavylium cationic form (ca. 96% at pH 1.5), since slow interconversion between the different chemical species at higher pH leads to severe peak broadening [24]. Moreover, under the acidic conditions, the anthocyanins absorb maximally at ca. 520 nm, leading to high sensitivity. Since the wine pigments are the only compounds absorbing in the region of 520 nm, this wavelength can be used for their selective detection and quantitation. This also means that, unless clear UV spectra are required for identification purposes, no sample preparation is needed for the complex wine sample and filtered wine samples can be directly injected. Formic acid was chosen to adjust the pH because of its volatility and strong acid characteristics. The acid content of phase A (water) and phase B (acetonitrile) was evaluated between 1 and 10%. At 1% formic acid (pH of phase A is 2.1) broad peaks were observed. The peak height increased with acid content up to 7.5% (pH 1.6), where it remained relatively stable. Therefore, pH 1.6 was chosen as the optimum acid content. The gradient was tuned as specified in the Section 2 to deliver optimal separation of wine anthocyanins within an acceptable

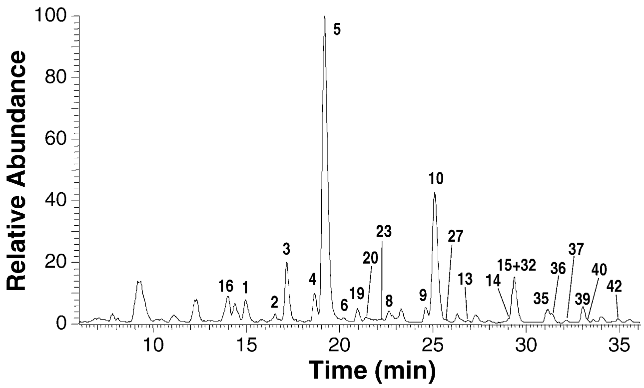


Fig. 1. Base peak chromatogram obtained from the LC–MS analysis of a South African red wine (Pinotage, 2001). Method details, see Section 2. Peak identification: see Table 2.

time. It can be noted that even when working at this low pH, no loss of separation efficiency was observed after months of analyses using the same column. Further, this mobile phase was selected as optimal for routine LC–UV analysis. While these conditions might not be ideally suited to MS detection, they proved adequate for the MS experiments reported here.

Due to a lack of available anthocyanin standards, external calibration was performed using malvidin-3-glucoside, and all other compounds were quantified using this calibration graph. Linearity was checked over the range 5–250 mg L^{-1} (ppm) (triplicate injections at five levels, $R^2 = 0.9996$), and the limit of detection (S/N = 3) was 0.18 ppm.

3.2. LC–MS identification of wine anthocyanins

Fig. 1 presents the base peak chromatogram obtained for a South African red wine. In this chromatogram, the base peaks are plotted against retention time. Representative mass spectra for a number of malvidin-derived pigments found in wine are presented in Fig. 2. Structures of the pigments identified in wines are presented in Table 1. Molecular ion and fragmentation information, together with λ_{max} values and relevant literature references for the identified compounds are presented in Table 2.

The predominant coloured species present in young red wines were those originating from the grape. Thus, the 3-glucoside derivatives of delphinidin, cyanidin, petunidin, peonidin and malvidin (**1–5**, Fig. 1) eluted in the specified order, with malvidin-3-glucoside being the major anthocyanin in all cases [24,30–32]. λ_{max} values were lower for cyanidin-glucoside and peonidin-glucoside compared to the other three, which is in agreement with reports by other authors [38]. Mass spectra contain the molecular ion $[M]^+$ as base peak, together with the fragment ion $[M-162]^+$, corresponding to the loss of the glucose moiety (Fig. 2A) [34–37]. Cyanidin-glucoside was present in only trace amounts in most South African wines, but MS–MS experiments clearly showed the same spectra.

The corresponding 3-acetyl-glucoside- (**6–10**) and 3-*p*-coumaroyl-glucoside derivatives (**11–15**) of the five specified anthocyanidins were identified in a similar way. The acetyl-glucoside-derivatives displayed, apart from the molecular ion, another peak $[M-204]^+$, representing the aglycone after the loss of an acyl group (Fig. 2B). Coumaroyl-glucoside derivatives displayed the molecular ion and the aglycone fragment at $[M-308]^+$ (loss of the coumaroyl-glucoside group, Fig. 2C). The elution order for each anthocyanidin is glucoside < acetyl-glucoside < coumaroyl-glucoside [24,30–32]. Coumaroyl-glucoside derivatives are additionally identified by a pronounced shoulder at ca. 310 nm in the UV spectrum, which is missing in both the other species [40]. Malvidin-caffeoyl-glucoside (**28**) was identified by its mass spectrum and retention time [35]. The molecular ion was detected at *m*/*z* 655, and the aglycone fragment at 331.

In addition to the main grape anthocyanins, a number of derived pigments were identified. A dimeric compound resulting from the direct condensation of malvidin-glucoside and catechin/epicatechin (compound **16**) was detected at 14 min, displaying the molecular ion at *m*/*z* 781 plus a fragment at *m*/*z* 619, corresponding to the loss of glucose. The occurrence of this compound in wine has been hypothesised by Somers [1], and more recently it has been detected in wine samples [36], with a similar retention time as reported here.

Pyranoanthocyanins resulting from reaction between anthocyanins and pyruvic acid were also identified. These adducts were detected only for those anthocyanins present in sufficient quantities: petunidin-glucoside (**17**), peonidin-glucoside (**18**) and malvidin-glucoside (**19**), and were identified by their MS spectra (containing a molecular ion 68 mass units greater than the respective glycosylated anthocyanins) and their retention times (eluting shortly after the respective anthocyanins) [35–37]. Also, λ_{max} values were significantly lower than for free anthocyanins (i.e., ca. 510 nm, compared to 527 nm) [9,11]. The structure of compound **19**, named vitisin A by Bakker et al. [9] has been elucidated previously, although different structures were proposed [9,11]. More recent data corroborate the structure proposed by Fulcrand et al. [11] as depicted in Table 1 [42,43]. The loss of glucose from vitisin A (*m*/*z* 399, Fig. 2D) was observed [10], while the same fragment was detected for petunidin-glucoside-pyruvic acid (**17**, *m*/*z* 385) in MS–MS experiments. Pyruvic acid derivatives of malvidin-acetyl-glucoside (acetylvitisin A, **23**) and malvidin-coumaroyl-glucoside (coumaroylvitisin A, **27**) were also detected at *m*/*z* values of 603 and 707, respectively. A fragment at *m*/*z* 399 was present for each of these compounds, resulting from loss of acyl- and coumaroyl-glucoside groups from **23** and **27**, respectively [10,37,42].

An additional pyranoanthocyanin (**20**) resulting from the cyclo-addition of acetaldehyde to malvidin-3-glucoside, referred to vitisin B by Bakker et al. [10], was found at a retention time of 21.4 min. The mass spectrum showed, in addition to the molecular ion peak at *m*/*z* 517, an aglycone fragment at *m*/*z* 355 [10,37]. Similar products resulting from addition of acetaldehyde to malvidin-acetyl-glucoside (acetylvitisin B,

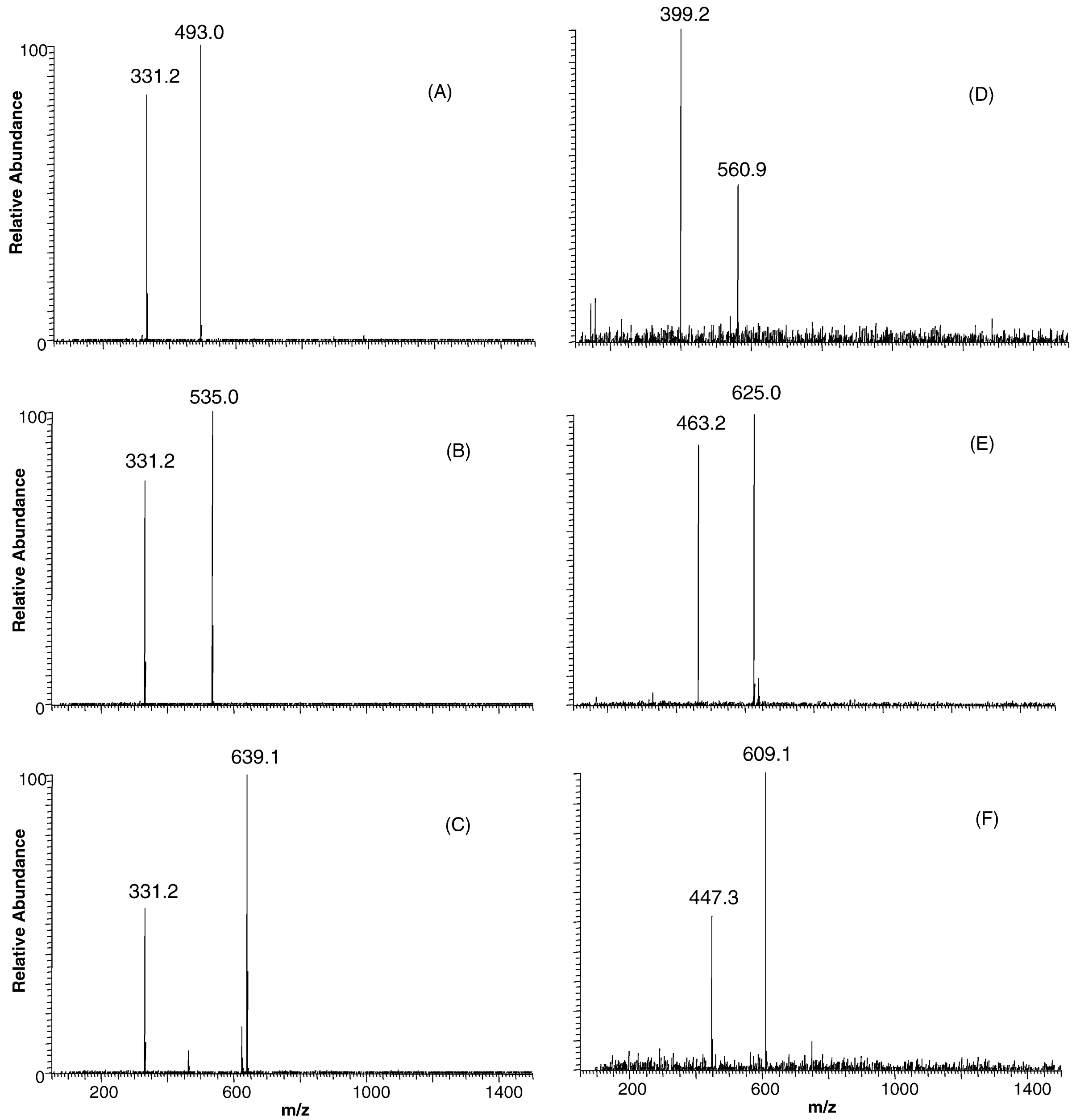


Fig. 2. Positive electrospray spectra of malvidin-derived pigments detected in red wine: (A) malvidin-3-glucoside (**5**); (B) malvidin-acetyl-glucoside (**10**); (C) malvidin-coumaroyl-glucoside (**15**); (D) vitisin A (**19**); (E) pinotin A (**32**); and (F) pigment A (**36**).

25) and malvidin-coumaroyl-glucoside (coumaroylvitisin B, **29**) were also detected. The mass spectra showed, apart from the molecular ion (*m*/*z* 559 and 663 for **25** and **29**, respectively), the same aglycone fragment as observed for vitisin B at *m*/*z* 355 [10,36].

Acetaldehyde-mediated condensation between malvidin-3-glucoside and (epi)catechin, leads to ethyl-bridged pigments [5]. Three of the possible isomers of these pigments were elucidated (**21, 24** and **26**), with mass spectra (molecular ion at *m*/*z* 809) and retention times in agreement with the literature [35–37]. A related compound (**22**), detected at *m*/*z* 1029, corresponds to a possible product of polymerisation reactions involving these ethyl-bridged pigments [35].

Compounds **30** and **33** are the catechin and epicatechin isomers of malvidin-3-glucoside-4-vinyl-catechin, previously detected in wine samples [44]. Similar compounds were detected in model solutions containing malvidin-3-glucoside, acetaldehyde and procyanidins [13], although none of the higher molecular weight pigments reported were detected in the present study. Mass spectra displayed the molecular ion at *m*/*z* 805, with fragments at *m*/*z* 643 representing the aglycones.

New pigments formed by addition of 4-vinylphenol to malvidin-glucoside and malividin-coumaroyl-glucoside have recently been reported in wine samples [6]. Both compounds, referred to as pigment A and B [6], as well as

Table 2
Retention times, mass spectral details and UV data of the anthocyanins identified in wine, together with cited references

No.	Rt	Compound	$[M]^+$	Fragments(MS)	Fragments (MS–MS)	λ_{max}	Reference(s)
1	15.0	Delphinidin-3-glucoside	465	303	n.p.**	527	[34–37]
2	16.4	Cyanidin-3-glucoside	449	287	449, 431, 287	517	[34–37]
3	17.1	Petunidin-3-glucoside	479	317	n.p.	527	[34–37]
4	18.7	Peonidin-3-glucoside	463	301	n.p.	516	[34–37]
5	19.2	Malvidin-3-glucoside (mv-3-glc)	493	331	n.p.	527	[34–37]
6	20.2	Delphinidin-(6-acetyl)-3-glucoside	507	303	n.p.	529	[34–37]
7	21.9	Cyanidin-(6-acetyl)-3-glucoside	491	287	n.p.	519	[34–37]
8	22.6	Petunidin-(6-acetyl)-3-glucoside	521	317	n.p.	529	[34–37]
9	24.6	Peonidin-(6-acetyl)-3-glucoside	505	301	n.p.	516	[34–37]
10	25.1	Malvidin-(6-acetyl)-3-glucoside	535	331	n.p.	529	[34–37]
11	26.6	Delphinidin-(6-coumaroyl)-3-glucoside	611	303	n.p.	527	[34–37]
12	n.d.*	Cyanidin-(6-coumaroyl)-3-glucoside	595	–	n.p.	–	[34–37]
13	26.9	Petunidin-(6-coumaroyl)-3-glucoside	625	317	n.p.	536	[34–37]
14	29.1	Peonidin-(6coumaroyl)-3-glucoside	609	301	n.p.	520	[34–37]
15	29.4	Malvidin-(6-coumaroyl)-3-glucoside	639	331	n.p.	517	[34–37]
16	14.0	Mv-3-glc-(epi)catechin	781	619	n.p.	–	[36]
17	18.4	Petunidin-3-glucoside-pyruvic acid	547	–	385	–	[35–37]
18	20.3	Peonidin-3-glucoside-pyruvic acid	531	507, 303	463	–	[35–37]
19	21.0	Vitisin A (mv-3-glc-pyruvic acid)	561	399	n.p.	509	[35–37,42]
20	21.4	Visitin B (mv-3-glc-acetaldehyde)	517	355	n.p.	–	[10,35–37]
21	21.7	Mv-3-glc-ethyl-(epi)catechin	809	–	None	–	[35–37]
22	21.8	Mv-3-glc-ethyl-(epi)catechin-unknown	1029	493, 331	None	–	[35]
23	22.3	Acetylvitisin A	603	399	n.p.	–	[10,42]
24	22.7	Mv-3-glc-ethyl-(epi)catechin	809	–	None	–	[35–37]
25	23.1	Acetylvisitin B	559	355	355	–	[10,36]
26	23.4	Mv-3-glc-ethyl-(epi)catechin	809	–	None	–	[35,37]
27	25.7	Coumaroylvitisin A	707	399	n.p.	–	[37,42,44]
28	26.0	Malvidin-(6-caffeoyl)-3-glucoside	655	331	None	–	[35]
29	26.6	Coumaroylvisitin B	663	355	None	–	[36]
30	27.1	Mv-glc-vinyl-catechin	805	643	n.p.	–	[44]
31	28.8	Pt-3-glc-4-vinylphenol	595	433	n.p.	–	[7]
32	29.3	Pinotin A (mv-3-glc-vinylcatechol)	625	463	n.p.	–	[45]
33	29.6	Mv-glc-vinyl-catechin	805	643	n.p.	–	[44]
34	30.9	Pt-3-acetyl-glc-4-vinylphenol	637	433	n.p.	–	[7]
35	31.2	Acetyl-pinotin A	667	463	n.p.	–	[7]
36	31.3	Pigment A	609	447	n.p.	503	[6,33,34]
37	32.0	Mv-3-glc-vinylguaiacol	639	477	n.p.	–	[7,44,46]
38	32.8	Pt-3-coumaroyl-glc-4-vinylphenol	741	433	n.p.	–	[7]
39	33.0	Coumaroyl-pinotin A	771	463	n.p.	–	[7]
40	33.2	Acetyl-pigment A	651	447	n.p.	–	[7,44]
41	33.8	Mv-acetyl-glc-vinylguaiacol	681	477	n.p.	–	[7]
42	34.9	Pigment B	755	447	n.p.	–	[6,44]
43	35.3	Mv-coumaroyl-glc-vinylguaiacol	785	477	n.p.	–	[7]
44	35.8	Mv-3-glc-vinylsyringol	669	–	n.p.	–	[7,45]

*, not detected; **, not performed.

the related compound acetyl-pigment A (malvidin-acetyl-glucoside-4-vinylphenol, **40**) were found in this study. The mass spectrum of pigment A (**36**) showed a molecular ion peak at *m*/*z* 609, while the loss of glucose led to the fragment detected at *m*/*z* 447 (Fig. 2F). The λ_{max} value for this compound is hypsochromically shifted to ca. 510 nm, compared to 527 nm for malvidin-glucoside, in accordance with the literature [6]. Pigment B (**42**) and acetyl-pigment A (**40**) displayed similar mass spectra, dominated by the molecular ions at *m*/*z* 755 and 651, respectively, and containing the same aglycone fragment at 447 in both cases [7,44].

Identical 4-vinylphenol adducts of petunidin-glucoside, petunidin-acetyl-glucoside and petunidin-coumaroyl-glucoside were detected (compounds **31**, **34** and **38**, respectively). Apart from the molecular ions (*m*/*z* 595, 637 and 741), an aglycone fragment was detected at *m*/*z* 433 for each of these compounds, as reported previously [7].

Recent evidence has suggested that the anthocyanin–vinylphenol adducts are in fact formed by direct reaction between intact cinnamic acids and anthocyanins (followed by decarboxylation) [45], as opposed to the previously proposed pathway involving free vinylphenols (resulting from enzymatic decarboxylation of cinnamic acids) [6,7]. Accordingly, the 4-vinylphenol adducts discussed above result from the reaction of various anthocyanins with *p*-coumaric acid. However, various related compounds have also been reported, resulting from the reaction of anthocyanins with ferulic, caffeic or synaptic acids present in red wines.

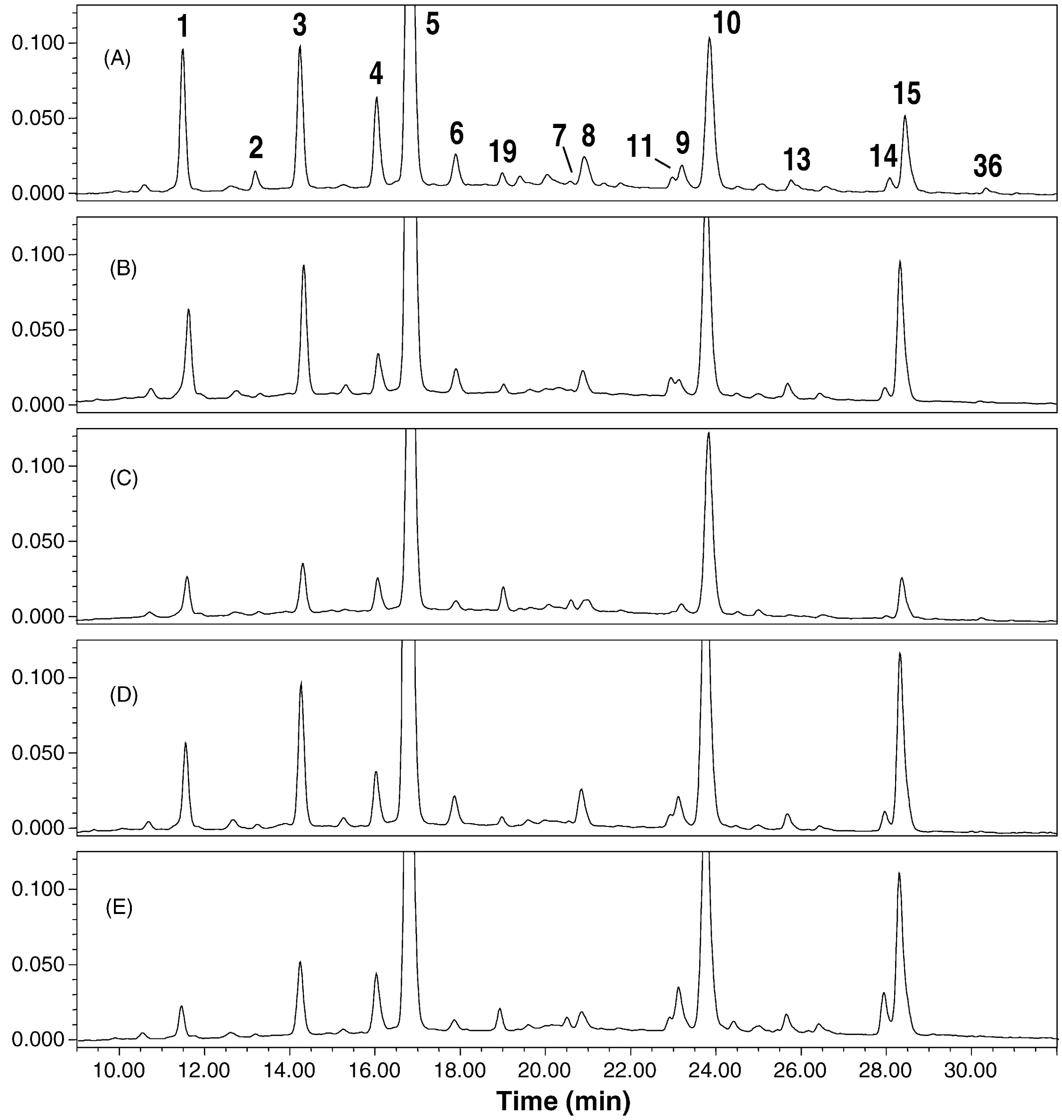


Fig. 3. Comparison between UV chromatograms obtained for five South African red wines. The 16 anthocyanins chosen for quantitation purposes are indicated. Peak identification: see Table 2. Wine cultivars: (A) Merlot; (B) Ruby Cabernet; (C) Cabernet Sauvignon; (D) Pinotage; and (E) Shiraz.

Thus, a new product resulting from the reaction of malvidin-3-glucoside and caffeic acid has recently been reported [8]. This compound is found in higher levels in Pinotage wines, a phenomenon ascribed to the higher levels of caffeic acid present in these wines [46], and for this reason named pinotin A. Pinotin A (**32**) and the related compounds acetyl-pinotin A (malvidin-acetyl-glucoside-vinylcatechol, **35**) and coumaroyl-pinotin A (malvidin-coumaroyl-glucoside-vinylcatechol, **39**) were also elucidated in the current study, prevalently in Pinotage wines. Aglycone fragments at *m*/*z* 463 were detected for each of these compounds (Fig. 2E).

The 4-vinyl-guaiacol derivatives of malvidin-3-glucoside (**37**), malvidin-acetyl-glucoside (**41**) and malvidin-coumaroyl-glucoside (**43**) were identified by their mass spectra, in accordance with [7], with aglycone fragments at *m*/*z* 477. These compounds result from the reaction between their respective anthocyanins and ferulic acid [45]. Similarly, the vinylsyringol adduct of malvidin-3-glucoside (**44**), resulting from the reaction between synaptic acid and malvidin-3-glucoside was detected at 35.8 min, with the molecular ion at *m*/*z* 669 dominating the mass spectrum.

3.3. Routine LC–UV analysis of wine anthocyanins

The power of LC–MS as identification tool for anthocyanins is evident from the precedent discussion. Chromatographic resolution of all the compounds listed in Table 2 was not achieved, and in fact was not required in order to identify even those compounds present in trace amounts. However, for routine and quantitative analysis, UV detection is often preferred because of simplicity, reliability and lower cost. For this reason, 16 compounds were selected, based on their prevalence in most wines, to be quantified by UV detection in

Table 3
ANOVA results for the anthocyanins in red wines (mean value for each variety and calculated *F* values)

Variety (*n*)	Cab Sauv 13	Merlot 10	Pinotage 11	Ruby Cab 10	Shiraz 11	F_{calc}
Delphinidin-3-glucoside (**1**)	2.1	20.2	7.7	14.4	3.8	8.8
Cyanidin-3-glucoside (**2**)	0.1	2.7	0.5	0.5	0.3	5.0
Petunidin-3-glucoside (**3**)	2.7	21.8	12.1	17.9	6.9	8.9
Peonidin-3-glucoside (**4**)	1.6	15.1	5.3	5.8	5.2	8.4
Malvidin-3-glucoside (**5**)	35.0	125.6	100.0	154.2	58.8	5.9
Delphinidin-(6-acetyl)-3-glucoside (**6**)	0.6	4.8	2.4	3.8	1.1	9.0
Vitisin A (**19**)	1.5	2.3	2.4	2.1	2.0	1.8
Cyanidin-(6-acetyl)-3-glucoside (**7**)	0.6	1.0	1.0	1.0	0.9	1.9
Petunidin-(6-acetyl)-3-glucoside (**8**)	0.7	5.9	3.5	3.6	1.9	8.8
Delphinidin-(6-coumaroyl)-3-glucoside (**11**)	0.1	1.6	0.6	2.1	0.5	9.3
Peonidin-(6-acetyl)-3-glucoside (**9**)	0.8	6.1	3.1	2.4	3.0	6.8
Malvidin-(6-acetyl)-3-glucoside (**10**)	14.2	37.8	31.2	36.1	18.5	3.1
Petunidin-(6-coumaroyl)-3-glucoside (**13**)	0.1	2.0	1.0	2.1	0.9	6.8
Peonidin-(6coumaroyl)-3-glucoside (**14**)	0.2	3.4	1.2	1.4	2.1	6.9
Malvidin-(6-coumaroyl)-3-glucoside (**15**)	4.5	18.2	15.1	20.7	9.9	5.7
Pigment A (**36**)	0.7	0.3	0.4	1.1	0.7	2.6

$F_{crit(5,54,0.05)=2.4)}$. Mean values are expressed as malvidin-3-glucoside equivalents, in mg L^{-1} (ppm). *Key:* Cab: Cabernet, Sauv: Sauvignon.

South African red wines. These compounds are specified in Fig. 3, and include, apart from the grape anthocyanins, also the derived products vitisin A and pigment A.

λ_{max} values, listed in Table 2, were used together with retention times for the confirmation of compounds. Quantitation of these 16 compounds was performed using UV detection. As discussed above, due to a lack of available anthocyanin standards, external calibration was performed using malvidin-3-glucoside, and all compounds were quantified using this calibration graph. Fig. 3 presents a comparison between LC–UV chromatograms obtained for the five South African cultivars of the same vintage (2003).

3.4. Classification of South African wines based on anthocyanin content

ANOVA results for the anthocyanins are presented in Table 3 together with mean values obtained for each compound in each of the cultivars. It should be noted that the mean values reported here could be somewhat misleading, since large variations in anthocyanin content for every cultivar were observed. This is a result of a rapid decrease in the anthocyanin levels with increasing age. Thus, varieties for which more young wines were analysed show higher mean amounts. At the chosen significance level of 95%, only the content of cyanidin-acetyl-glucoside and vitisin A did not differ significantly between varieties. Also, there was no significant difference in the anthocyanin composition between Shiraz and Pinotage, or Shiraz and Cabernet Sauvignon.

In stepwise forward LDA, the following variables were not included in the classification model: delphinidin-, cyanidin-, petunidin- and peonidin-glucosides, cyanidin-(6-acetyl)-3-glucoside and peonidin-(6-acetyl)-3-glucoside. The remaining 10 variables were used to obtain a classification function, which allowed the correct prediction of 80.0% of the wines according to variety. Since the training data set (the data set used to derive the classification function) was used to evaluate the classification of the LDA model, this is referred to as the recognition ability of the model. The results are presented in the classification matrix depicted in Table 4. Here, each wine is classified as belonging to the group where the value of its classification function is the largest. A scatter plot of wine samples in the plane defined by the first two canonical roots is presented in Fig. 4. While good discrimination is observed between Merlot, Ruby Cabernet and Pinotage wines, the close

Table 4
Classification matrix obtained by stepwise forward LDA for the anthocyanin data

Group	Percent	Cab Sauv	Merlot	Pinotage	Ruby Cab	Shiraz
Cab Sauv	100.0	13	0	0	0	0
Merlot	80.0	2	8	0	0	0
Pinotage	90.9	1	0	10	0	0
Ruby Cab	80.0	1	0	1	8	0
Shiraz	45.5	6	0	0	0	5
Total	80.0	23	8	11	8	5

Rows represent observed classifications.

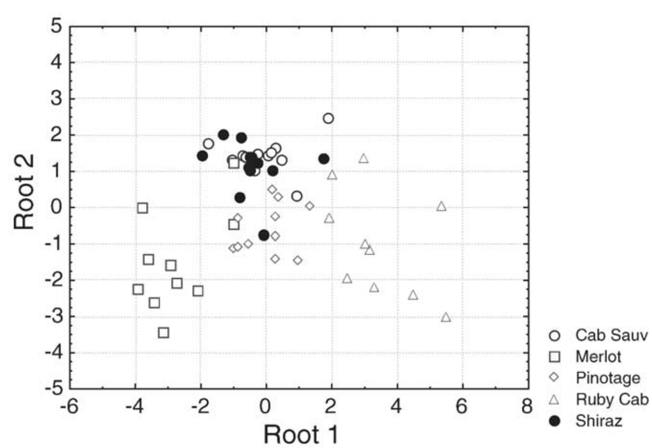


Fig. 4. Scatter plot of canonical scores on the plain defined by the first two canonical roots, obtained from the anthocyanin data for red wines.

proximity of Shiraz wines to Cabernet Sauvignon reflects the poor discriminating power of the model towards these wines, as is evident from the classification matrix (Table 4).

The poor recognition capabilities of the model are somewhat surprising in view of literature results reported for the anthocyanins. The broad range of vintages analysed in the current study may be partially to blame, by obscuring cultivar-related differences. This may be explained in light of the fact that grape anthocyanins decrease rapidly during wine ageing as they are replaced by more stable derived pigments [1], a process that might lead to disruptions of the anthocyanin pattern over the extended time period studied here. Recent work in progress in our laboratory indicates that the non-coloured phenolic content is more suited to the differentiation of wines.

4. Conclusions

A HPLC–MS method was developed and used to identify a total of 44 pigments in South African wines, including grape anthocyanins and pigments derived during ageing. Based on these results, an LC–UV method suitable for the routine analysis of 16 wine anthocyanins is proposed. Direct injection of filtered wine samples followed by selective detection at 520 nm allowed quantitation of these compounds in a wide variety of South African red wines. The LC method has the advantages of being rapid, reproducible and sensitive, making it the ideal tool for the characterisation of wines by their anthocyanin pattern. Significant variation in anthocyanin content for a given cultivar, due to wine ageing, was observed. Recent work showed that the comparison of non-coloured phenolics is more reliable for wine differentiation.

References

[1] T.C. Somers, Phytochemistry 10 (1971) 2175.
[2] E. Haslam, Phytochemistry 19 (1980) 2577.
[3] H. Liao, Y. Cai, E. Haslam, J. Sci. Food Agric. 59 (1992) 299.
[4] S. Remy, H. Fulcrand, B. Labarbe, V. Cheynier, M. Moutounet, J. Sci. Food Agric. 80 (2000) 745.
[5] H. Fulcrand, T. Doco, N.E. Es-Safi, V. Cheynier, M. Moutounet, J. Chromatogr. A 752 (1996) 85.
[6] H. Fulcrand, P.J. Cameira dos Santos, P. Sarni-Manchado, V. Cheynier, J. Favre-Bonvin, J. Chem. Soc. Perkin Trans. 1 (1996) 735.
[7] Y. Hayasaka, R.E. Asenstorfer, J. Agric. Food Chem. 50 (2002) 756.
[8] M. Schwarz, G. Jerz, P. Winterhalter, Vitis 42 (2003) 105.
[9] J. Bakker, P. Bridle, T. Honda, H. Kuwano, N. Saito, N. Terahara, C.F. Timberlake, Phytochemistry 44 (1997) 1375.
[10] J. Bakker, C.F. Timberlake, J. Agric. Food Chem. 45 (1997) 35.
[11] H. Fulcrand, C. Benabdeljalil, J. Rigaud, V. Cheynier, M. Moutounet, Phytochemistry 47 (1998) 1401.
[12] C. Romero, J. Bakker, J. Agric. Food Chem. 47 (1999) 3130.
[13] E.M. Francia-Aricha, M.T. Guerra, J.C. Rivas-Gonzalo, C. Santos-Buelga, J. Agric. Food Chem. 45 (1997) 2262.
[14] T.C. Somers, M.E. Evans, J. Sci. Food Agric. 25 (1975) 1369.
[15] J. Bakker, P. Bridle, S.J. Bellworthy, C. Garcia-Viguera, H.P. Reader, S.J. Watkins, J. Sci. Food Agric. 78 (1998) 297.
[16] M.P. Kähkönen, J. Heinämäki, V. Ollilainen, M. Heinonen, J. Sci. Food Agric. 83 (2003) 1403.
[17] E. García-Beneytez, E. Revilla, F. Cabello, Eur. Food Res. Technol. 215 (2002) 32.
[18] P. Etiévant, P. Schlich, A. Bertrand, P. Symonds, J.C. Bouvier, J. Sci. Food Agric. 42 (1988) 39.
[19] B. Berente, D. De La Calle García, M. Reichenbächer, K. Danzer, J. Chromatogr. A 871 (2000) 95.
[20] I. Arozarena, A. Casp, R. Marín, M. Navarro, Eur. Food Res. Technol. 212 (2000) 108.
[21] S. Kallithraka, I.S. Arvanitoyannis, P. Kefalas, A. El-Zajouli, E. Soufleros, A. Psarra, Food Chem. 73 (2001) 501.
[22] S. Frías, J.E. Conde, F. Rodríguez-Bencomo, F. García-Montelongo, J.P. Pérez-Trujillo, Talanta 59 (2003) 335.
[23] E. Marengo, M. Aceto, Food Chem. 81 (2003) 621.
[24] L.W. Wulf, C.W. Nagel, Am. J. Enol. Vitic. 29 (1978) 42.
[25] T.C. Somers, M.E. Evans, J. Sci. Food Agric. 28 (1977) 279.
[26] J. Bakker, N.W. Preston, C.F. Timberlake, Am. J. Enol. Vitic. 37 (1986) 121.
[27] R. Sáenz-Lopez, P. Fernández-Zurbano, M.T. Tena, J. Chromatogr. A 990 (2003) 247.
[28] H. Morais, C. Ramos, E. Forgács, T. Cserháti, J. Oliviera, J. Chromatogr. B 770 (2002) 297.
[29] J.P. Goiffon, P.P. Mouly, E.M. Gaydou, Anal. Chim. Acta 382 (1999) 39.
[30] T.V. Johnston, J.R. Morris, J. Food Sci. 62 (1997) 684.
[31] B. Holbach, R. Marx, M. Ackermann, Lebensmittelchemie 51 (1997) 78.
[32] E. Mataix, M.D. Luque de Castro, J. Chromatogr. A 910 (2001) 255.
[33] W. Mullen, M.E.J. Lean, A. Crozier, J. Chromatogr. A 966 (2002) 63.
[34] A. Baldi, A. Romani, N. Mulinacci, F.F. Vincieri, B. Casetta, J. Agric. Food Chem. 43 (1995) 2104.
[35] I. Revilla, S. Pérez-Magariño, M.L. González-SanJosé, J. Chromatogr. A 847 (1999) 83.
[36] A.M. Vivar-Quintana, C. Santos-Buelga, J.C. Rivas-Gonzaldo, Anal. Chim. Acta 458 (2002) 147.
[37] V. Atanasova, H. Fulcrand, V. Cheynier, M. Moutounet, Anal. Chim. Acta 458 (2002) 15.
[38] A. Piovan, R. Filippini, D. Favretto, Rapid Commun. Mass Spectrom. 12 (1998) 361.
[39] J.A. Sugui, K.V. Wood, Z. Yang, C.C. Bonham, R.L. Nicholson, Am. J. Enol. Vitic. 50 (1999) 199.
[40] V. Hong, R.E. Wrolstad, J. Agric. Food Chem. 38 (1990) 708.
[41] B.R. Kowalski, C.F. Bender, J. Am. Chem. Soc. 94 (1972) 5632.
[42] N. Mateus, A.M. Silva, J. Vercauteren, V. de Freitas, J. Agric. Food Chem. 49 (2001) 4836.
[43] M. Schwarz, P. Quast, D. von Baer, P. Winterhalter, J. Agric. Food Chem. 51 (2003) 6261.
[44] R.E. Asenstorfer, Y. Hayasaka, G.P. Jones, J. Agric. Food Chem. 49 (2001) 5957.
[45] M. Schwarz, T.C. Wabnitz, P. Winterhalter, J. Agric. Food Chem. 51 (2003) 3682.
[46] M. Schwarz, G. Hofmann, P. Winterhalter, J. Agric. Food Chem. 52 (2004) 498.