



Simultaneous separation by reversed-phase high-performance liquid chromatography and mass spectral identification of anthocyanins and flavonols in Shiraz grape skin

Mark O. Downey^{a,*}, Simone Rochfort^b

^a Viticulture, Future Farming Systems Research, Department of Primary Industries Victoria, P.O. Box 905, Mildura, Vic. 3502, Australia

^b Discovery Technologies, Biosciences Research, Department of Primary Industries, 621 Sneydes Road, Werribee, Vic. 3030, Australia

ARTICLE INFO

Article history:

Received 1 April 2008

Received in revised form 27 May 2008

Accepted 2 June 2008

Available online 6 June 2008

Keywords:

Anthocyanin

Flavonol

Flavonoid

HPLC

Mass spectrometry

ABSTRACT

A limitation of large-scale viticultural trials is the time and cost of comprehensive compositional analysis of the fruit by high-performance liquid chromatography (HPLC). In addition, separate methods have generally been required to identify and quantify different classes of metabolites. To address these shortcomings a reversed-phase HPLC method was developed to simultaneously separate the anthocyanins and flavonols present in grape skins. The method employs a methanol and water gradient acidified with 10% formic acid with a run-time of 48 min including re-equilibration. Identity of anthocyanins and flavonols in Shiraz (*Vitis vinifera* L.) skin was confirmed by mass spectral analysis.

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1. Introduction

Reversed-phase high-performance liquid-chromatography (HPLC) has long been used for the analysis of anthocyanins and flavonols in plants and plant products, including grapes and wine. Generally, a separate analytical method has been required for the analysis of anthocyanins and flavonols [1], although some methods do purport to separate multiple classes of flavonoids simultaneously [2–4]. Of the many published HPLC methods for separating flavonols and anthocyanins, most are unsatisfactory for a range of reasons, first among these is the poor resolution of peaks, particularly of peonidin and malvidin derivatives [4–6]. In addition, many of the methods have lengthy run-times, often greater than 1 h [7–9]. This reduces sample throughput and subsequently limits the size of many experiments, long run-times also increase solvent consumption and the analytical cost per sample.

Peak resolution has been improved through the selection of mobile phases such as perchloric acid [8,10,11], however the highly corrosive nature of perchloric acid damages metallic parts of the HPLC. The use of highly corrosive or strong organic solvents in the mobile phase also creates a substantial volume of toxic waste

requiring costly disposal. Thus, we sought to develop a HPLC method that utilised relatively innocuous solvents and gave good peak resolution of both anthocyanins and flavonols that improved on previous run-times for routine analysis of grape and wine samples.

2. Experimental

2.1. Materials and reagents

Standard materials, standards of malvidin-3-O-glucoside, cyanidin-3-O-glucoside and quercetin-3-O-glucoside were obtained from Extrasynthase, France. All other chemicals and reagents were analytical-reagent or HPLC grade.

2.2. Grape samples

Fruit from the wine grape (*Vitis vinifera* L.) cultivar Shiraz (syn. Syrah) were harvested from a commercial vineyard in Sunraysia, northwest Victoria, Australia (34°27' south, 142°19' east), during the 2003 vintage. Approximately 20 bunches were collected across 10 panels when the fruit reached a sugar level of 24.5°Brix. Grape berries were weighed and the skins were removed and weighed. Grape skins were then frozen in liquid nitrogen, ground to a fine powder, and stored at –80 °C until analysed.

* Corresponding author. Fax: +61 3 50514523.

E-mail address: mark.downey@dpi.vic.gov.au (M.O. Downey).

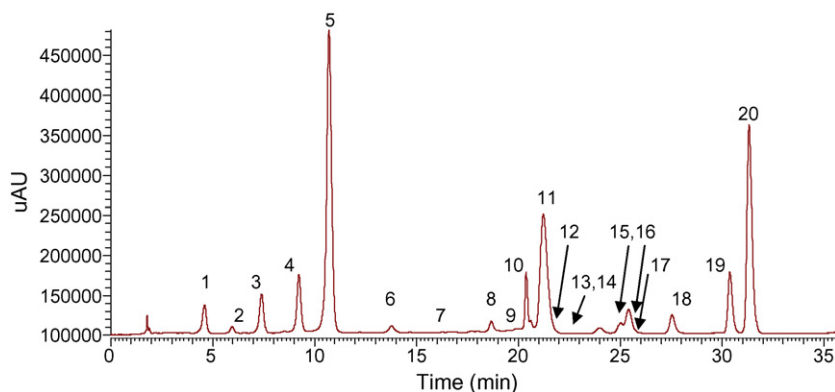


Fig. 1. HPLC chromatogram of Shiraz skin extract at 520 nm. See Table 1 for compound identification.

2.3. Extraction of anthocyanins and flavonols

Anthocyanins and flavonols were extracted from grape skins in three analytical replicates. The extraction technique used has been optimised for extraction of anthocyanins and flavonols and has been reported in an earlier study [12]. In brief: Three aliquots of 0.1000 g (± 0.0005 g) of frozen ground grape skin were extracted in 1.0 mL of 50% (v/v) methanol in water for 20 min with sonication [12]. The extracts were then centrifuged (10 min at 13,000 \times g) and 200 μ L of the supernatant transferred to HPLC auto-sampler vials. The injection volume for each analysis was 25 μ L.

2.4. HPLC separation of anthocyanins and flavonols

HPLC separation of anthocyanins and flavonols from grape skin utilised 10% formic acid in water (Solvent A) with a 10% formic acid in methanol (Solvent B) gradient at a flow rate of 1.0 mL/min. The column temperature was maintained at 40 °C for the duration of the analysis. The column selected was a C-18 SS Wakosil (150 mm \times 4.6 mm, 3 μ m packing; SGE, Ringwood, Australia) protected by an SGE C-18 guard column. Samples were run on a HP1100 (Agilent, Mulgrave, Australia) HPLC system. The gradient conditions were: 0 min, 18% B; 14 min, 29% B; 16 min, 32% B; 18 min, 41% B;

Table 1
UV and MSⁿ data of anthocyanins

No.	Compound	Elution time (min)	λ_{\max} (nm)	m/z [M+H] ⁺	Mass loss (M+H ⁺)-MS ²	MS ²	MS ^{3a}	MS ^{4a}
1	Delphinidin-3-O-glucoside	4.53	526	465	−162	303	229, 257, <u>303</u>	229, 257
2	Cyanidin-3-O-glucoside	5.78	520	449	−162	287	213, 231, 241, 259, <u>287</u>	213, 231, 241, 259, <u>287</u>
3	Petunidin-3-O-glucoside	7.16	344, 526	479	−162	317	257, 274, <u>302</u> , 317	218, 228, 246, <u>274</u>
4	Peonidin-3-O-glucoside	9.04	520	463	−162	301	286	230, 258, 268
5	Malvidin-3-O-glucoside	10.47	348, 528	493	−162	331	179, 242, 270, 287, 299, <u>315/316</u>	213, 257, 285, 287, 313, 315
6	Delphinidin-3-O-acetylglucoside	13.05	530 ^b	507	−204	303	229, 257, <u>303</u>	229, 257, <u>303</u>
7	Cyanidin-3-O-acetylglucoside	16.34	na	491	−204	287	213, 231, 259, <u>287</u>	213, 231, 259, <u>287</u>
8	Petunidin-3-O-acetylglucoside	17.9	350, 530	521	−204	<u>302</u> , 317	218, 228, 246, 256, <u>274</u>	135, 149, 153, 163181
9	Peonidin-3-O-acetylglucoside	20.2	522	505	−204	301	286	230, 258, 268
10	Petunidin-(6-O-caffeoyl)glucoside	20.34	na	641	−324	317	302	218, 228, 246, 274
11	Malvidin-3-O-acetylglucoside	20.86	344, 532	535	−204	331	179, 242, 270, 287, 299, <u>315/316</u> , 331	257, 285, 287, 313, 315
12	Delphinidin-3-(6-O-coumaroyl)glucoside (<i>cis</i> isomer)	21.04	na	611	−308	303	229, 257, <u>303</u>	229, 257, 303
13	Peonidin-(6-O-caffeoyl)glucoside	22.96	na	625	−324	301	258, <u>286</u>	230, 258, 268
14	Peonidin-3-(6-O-coumaroyl)glucoside (<i>cis</i> isomer)	23.03	na	609	−308	301	286	230, 258, 268
15	Malvidin-3-(6-O-coumaroyl)glucoside (<i>cis</i> isomer)	24.90	538	639	−308	331	179, 242, 270, 287, 299, <u>315/316</u> , 331	257, 285, 287, 313, 315
16	Malvidin-(6-O-caffeoyl)glucoside	25.26	324, 532	655	−324	331	179, 242, 270, 287, 299, <u>315/316</u> , 331	257, 285, 287, 313, 315
17	Cyanidin-(6-O-coumaroyl)glucoside (<i>trans</i> isomer)	25.35	na	595	−308	287	213, 231, 259, <u>287</u>	213, 231, 259, <u>287</u>
18	Petunidin-(6-O-coumaroyl)glucoside (<i>trans</i> isomer)	27.17	534	625	−308	317	274, <u>302</u>	218, 228, 246, <u>274</u>
19	Peonidin-3-(6-O-coumaroyl)glucoside (<i>trans</i> isomer)	30.05	312, 522	609	−308	301	286	230, 258, 268
20	Malvidin-3-(6-O-coumaroyl)glucoside (<i>trans</i> isomer)	31.04	318, 534	639	−308	331	179, 242, 270, 287, <u>299</u> , <u>315/316</u> , 331	225, 253, <u>281</u> , 299

^a Base peak (100%) is underlined.

^b Overlapping peak, na = data not available due to lack of signal intensity.

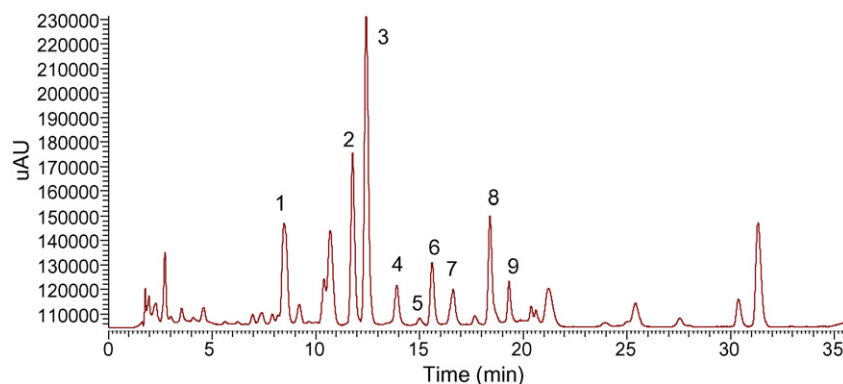


Fig. 2. HPLC chromatogram of Shiraz skin extract at 353 nm. See Table 2 for compound identification.

18.1 min, 30% B; 29 min, 41% B; 32 min, 50% B; 34.5 min, 100% B; 35–38 min, 18% B.

The method is robust and reproducible, demonstrating linearity for the standards quercetin-3-glucoside over four orders of magnitude (0.0001–1 mg/mL, $R^2 = 0.9994$) and for malvidin-3-glucoside over two orders of magnitude (0.05–1 mg/mL, $R^2 = 0.9982$).

2.5. Identification of anthocyanins and flavonols

Anthocyanins were monitored by photodiode array detection (DAD) at 520 nm and flavonols monitored at 353 nm [13]. Anthocyanins and flavonols were putatively identified by comparison of their spectra with commercial standards of malvidin-3-O-glucoside, cyanidin-3-O-glucoside and quercetin-3-O-glucoside and of their elution order with previously published separations [5,6]. The method was then transferred to a HP1100 HPLC system equipped with a Thermo Scientific LTQ linear ion trap for mass spectral (MS) analysis and confirmation of anthocyanin and flavonol identity.

For LC–MS/MS experiments, an Agilent 1100 series HPLC system comprising a quaternary gradient pump, autosampler and diode array detector was coupled to a Thermo Electron LTQ ion trap mass spectrometer (Waltham, MA, USA). Extract (50 μ L) was injected onto the same column utilising the same elution conditions as described above.

2.5.1. Mass spectrometric acquisition parameters

For LC–MS/MS experiments a data dependent protocol was used either with electrospray ionization (ESI) positive mode or ESI negative mode with a mass range of 150–1500 units. Dynamic exclusion was engaged with a 60-s exclusion time. Data were acquired using automated MS/MS settings with a target of 30,000, a normalised collision energy of 35 and an ion max time of

200 ms. The heated capillary was maintained at 270 °C and the sheath, auxiliary and sweep gases were at 65, 20 and 14 units, respectively. Source voltage was set to 4.4 kV with a capillary voltage of 25 V for positive data acquisition and source voltage of 3.4 kV and capillary voltage –43 V for negative data acquisition. Prior to data acquisition the system was tuned using a 250 μ g/mL standard of rutin. The rutin was infused via syringe pump through a T-piece at a rate of 15 μ L/min with an HPLC flow rate of 1 mL/min and a solvent composition of 50% A and 50% B.

3. Results and discussion

3.1. HPLC separation of anthocyanins and flavonols

The grape skin extracts were analysed on a reversed-phase C-18 column with the mobile phase containing 10% formic acid. Formic acid is a common modifier for reversed-phase HPLC and its volatility also makes it highly suitable for mass spectrometry. In general most LC–MS applications employ this modifier in the range 0.1–1%. The high concentration employed in this methodology was necessary to ensure the good peak shape and resolution that can be obtained with lower levels of more corrosive acids such as perchloric acid. The extracts contained a large number of flavonoids. The anthocyanin class was readily identifiable from their UV λ_{\max} near 520 nm and a chromatogram extracted at 520 nm from the DAD data clearly showed these metabolites. Twenty anthocyanins were identified, and the major compounds are all well resolved (Fig. 1, Table 1). The flavonols were monitored at a wavelength of 353 nm (Fig. 2) though it must be noted that other metabolites also absorb at this wavelength, including many of the anthocyanins. Nine flavonols were identified, all of which were well resolved.

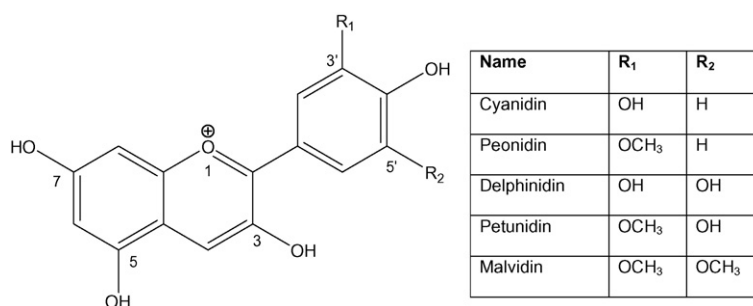


Fig. 3. General anthocyanidin structure. Glycosylation at the 3 position produces the anthocyanin series shown in the table.

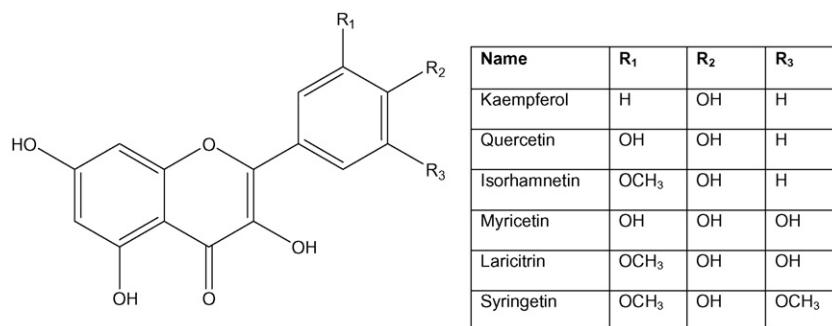


Fig. 4. Core subunits of flavonols found in wine.

3.2. Confirmation of anthocyanins and flavonols by LC–MS/MS

Anthocyanins are pigments that give colour to many plants including grapes. They occur naturally as glycosides and have the general structure shown in Fig. 3. The glycosylation pattern in grapes can be complex, however, it has been shown that for cultivars of *V. vinifera* glycosylation appears exclusively at the 3 position. This is not the case for non-*Vitis* or hybrid grapes where glycosylation at both the 3 and 5 positions is common [14–16]. In this study the identity of anthocyanins and flavonols in Shiraz (*V. vinifera* L.) skin was confirmed by mass spectral analysis which included MS² to MS⁴ fragmentation (Table 1). The anthocyanins were detected in positive mode with the [M+H]⁺ ion subjected to further fragmentation to allow identification of the anthocyanidin (aglycone) core. MS³ to MS⁴ fragmentations were useful in confirming the core since the fragment ions of common aglycones are consistent (Table 1). The elution of the anthocyanins were consistent across each series of derivatives with delphinidin, the most polar anthocyanidin, eluting first followed by the cyanidin, petunidin, peonidin and malvidin derivatives. The 3-*O*-glycosides (1–5) elute first followed by the acetylglucosides (6–9 and 11), the caffeoylglycosides (10, 13 and 16) and the coumaroylglycosides (*cis*: 12, 14, 15; *trans*: 17–20); fully consistent with previously reported data [15–19]. The derivatives were assigned based on the neutral loss from the parent ion: glycoside (–162), acetylglucose (–204), coumaroylglycoside (–308) and caffeoylglycoside (–324) (Table 1). Both the *cis* and *trans* isomers of the coumaroylglycosides were observed for peonidin and malvidin. The *cis* isomers were identified by their earlier elu-

tion times [11,18], and in this study they eluted more than 5 min before the *trans* analogues. Only the *cis* analogue was observed for delphinidin (12), albeit present in minor amounts (see Fig. 1) and it was identified based on its retention time compared to that of the peonidin and malvidin *cis*-coumaroylglycosides. Monagas et al. [18] report that the *trans* coumaroylglycoside of malvidin converts to the *cis* isomer after extended exposure to UV light. The possibility that any *trans*-coumaroyldelphinidin that may have been present was converted completely to the *cis* form cannot be ruled out.

The acyl substitution of anthocyaninglycosides is generally reported in the *trans* form [20,21]. Reports of *cis* isomers are not widespread being first observed in purple leaves of *Perilla ocimoides* [22] and subsequently by several other authors [20,23,24] including García-Beneytez et al. [11] and Monagas et al. [18] in grapes and wine. The early work by Yoshida et al. [22] showed that *in vitro* isomerisation occurred under both artificial light (high-pressure Hg lamp: 366 nm) and direct sunlight irradiation and suggested that similar isomerisation may occur in sun-exposed leaves. Subsequently, it was demonstrated that this isomerisation occurred *in vivo* in the irradiated flowers *Petunia integrifolia* and *Tritelia bridge-seii* [25]. Thus, it seems likely that the presence of *cis* isomers of anthocyanin coumaroyl glucosides in Shiraz grape skin occur as a result of sun exposure.

Like anthocyanins, flavonols tend to occur naturally as glycosides and have the general structure shown in Fig. 4. Myricetin, quercetin, laricitrin, kaempferol, isorhamnetin and syringetin have all been detected in red skin grapes and the concentrations vary according not only to variety but also environmental

Table 2
MS data of flavonols

No.	Compound	Elution time (min)	λ_{\max} (nm)	m/z [M–H] [–]	MS ^{2a}	MS ^{3a}	MS ^{4a}
1	Myricetin-3- <i>O</i> -glucoside	8.37	350	479	316/317	242, 270/271, 287	171, 199, 227
2	Quercetin-3- <i>O</i> -glucuronide	11.55	352	477	301	151, 179, 193, 257, 273	151
3	Quercetin-3- <i>O</i> -glucoside	12.16	352	463	301	151, 179, 193, 257, 273	151
4	Laricitrin-3- <i>O</i> -galactoside ^c	13.57	354	493	330, 331	151, 179, 193, 316, 317	151, 164, 179, 219, 244, 270/271, 287/288
5	Kaempferol-3- <i>O</i> -glucoside	14.69	352	447	255, 284/285, 327, 401, 419, 429	227, 239, 255/256	212, 227
6	Laricitrin-3- <i>O</i> -rhamnose-7- <i>O</i> -trihydroxycinnamic acid ^{b,c}	15.30	314	655	303, 314, 329, 347, 475, 501, 509	314	299
7	Kaempferol-3- <i>O</i> -caffeoylate ^{b,c}	16.30	322	447	284/285	227, 239, 255/256	212, 227
8	Isorhamnetin-3- <i>O</i> -glucoside	18.06	354	477	271, 285, 314/315, 357	243, 257, 271, 285/286, 299/300	241/270
9	Syringetin-3- <i>O</i> -galactoside ^c	19.40	358	507	344/345, 387, 479, 489	330	

^a Base peak (100%) is underlined.

^b Previously unidentified metabolites.

^c Tentative structure only.

conditions such as sun exposure [2,26–28]. Sugar substitution on flavonols usually occurs as the *O*-glycosides, mainly at the 3-, 7-, and 4'-positions and previous studies indicate that the UV spectrum is indicative of substitution position [29,30]. Inspection of the UV spectra for the nine compounds (Table 2) suggests that the 3-hydroxyl group is blocked (maxima, 347–355 nm), indicating that there is substitution at this position. Similarly, the UV spectra of the flavonols suggested that several of these metabolites were acylated with hydroxycinnamic acid derivatives, through the presence of a broad maximum present around 320 nm (Table 2). Myricetin-3-*O*-glucoside (1), quercetin-3-*O*-glucoside (2), quercetin-3-*O*-glucuronide (3), kaempferol-3-*O*-glucoside (5) and isorhamnetin-3-*O*-glucoside (8) were readily identified by comparison of the previously reported MS fragmentation data for these metabolites [1] and from inspection of the UV spectra, with the maxima at around 350 nm confirming the glycoside substitution at the 3 position. The less common metabolites laricitrin-3-*O*-glycoside (4) and syringetin-3-*O*-glycoside (9) were identified in a similar manner. The UV-max indicated that there was substitution at the 3 position, whilst the MS fragmentation data (loss of 162 units) suggested that a hexose sugar was the substituent. Mass spectrometry does not allow the differentiation between glucose and galactose substituents. However, it has been suggested that for laricitrin and syringetin the galactose substituents are more common, whilst the converse is true for the other flavonols [1,28,31,32] and on this basis we have tentatively assigned the metabolites as noted in Table 2.

Compound 7 has been tentatively identified as kaempferol-3-caffeoylate on the basis of its UV-max at 322 nm which is indicative of substitution by a hydroxycinnamic acid derivative and its significantly later elution time (almost 2 min) than the glycoside analogue (compound 5). This is the first report of this metabolite in grape skin, although the related metabolite 3-*O*-*trans*-*p*-coumaroylkaempferol has been reported from a number of plants including grapes [33]. Compound 6 is another unusual metabolite that has been tentatively identified as laricitrin-3-*O*-rhamnose-7-*O*-trihydroxycinnamic acid. The UV-max of this metabolite suggested the presence of the hydroxycinnamic acid derivative whilst the placement of the substituents is suggested by the MS² spectrum. It is more common for the hydroxycinnamic acid derivatives to be attached to the sugar moiety rather than the flavonol core, and the loss of the hydroxycinnamic acid, or loss of the acid with the glycoside, is seen in the MS² scan event as the only fragment ions [29]. This is similar to the way in which the hydroxycinnamic acid derivatives of the anthocyanins were observed to fragment in this study (Table 1). The fragmentation is markedly different for compound 6 with ions corresponding [M-sugar][−] (*m/z* 509) and [M-trihydroxycinnamic acid][−] (*m/z* 475) both present as well as the [M-sugar-trihydroxycinnamic acid][−] ion (*m/z* 329). Again, this is the first report of this metabolite in grapes although the related metabolite kaempferol-7-*p*-coumaroyl-3-glucoside has been reported in wine [34].

The metabolites 6 and 7 appear to be novel natural products and to confirm their identity the compounds should be fully characterised by additional techniques including NMR spectroscopy. This was beyond the scope of this study and so the structure assignments of 6 and 7 must be considered tentative.

4. Conclusion

Here we have presented an analytical method that simultaneously separates a wide range of anthocyanins and flavonols. We have confirmed the identity of the flavonoids observed in the

separation by LC–MS/MS and using MS^{*n*} (*n* = 2–4, sequential MS fragmentation events) techniques tentatively identified two novel compounds. While the role of flavonols has not been conclusively determined, anthocyanins are important in the colour of red wine, contributing wholly to the colour of young wines and through interactions with tannins and other wine components form pigmented polymers and modified anthocyanins that are responsible for the colour of mature wines. Nevertheless, flavonols may yet be determined to have a role in wine quality and flavonol analysis in grape berries is a useful contribution to furthering our understanding of flavonoid biosynthesis and the regulation of flavonoid biosynthesis in grapevines, as indeed is anthocyanin analysis. In our hands this method has proven robust and reliable for routine analysis of anthocyanin and flavonol analysis in winegrapes.

Acknowledgements

The authors gratefully acknowledge the expert technical assistance of Marica Mazza in the preparation of grape samples used in the development of this method. This research was supported by the Victorian Department of Primary Industries and by the grape-growers and winemakers of Australia through their investment body the Grape and Wine Research and Development Corporation.

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