It is the paper published as:

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Title: Copigmentation and anti-copigmentation in grape extracts studied by spectrophotometry and post-column-reaction HPLC

Journal: Food Chemistry **ISSN**: 0308-8146

Year: 2012 **Volume:** 132 **Issue:** 4 **Pages:** 2194-2201

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URLs: http://researchoutput.csu.edu.au/R/-?func=dbin-jump-full&object_id=36952&local_base=GEN01-CSU01
http://dx.doi.org/10.1016/j.foodchem.2011.12.058

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Copigmentation and anti-copigmentation in grape extracts studied by spectrophotometry and post column reaction HPLC

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ABSTRACT

Copigmentation is a very important contributor to color in young red wines. Further understanding of the copigmentation effect has been elucidated by investigating the contribution of various fractions of grapes – skin, pulp, and seed – in varieties Shiraz, Sangiovese and Pinot Noir. A series of spectrophotometric and HPLC experiments elucidated the effect of this interaction in grape extracts simulating wine conditions. A post column reaction method was developed to permit the evaluation of the reactivity of each grape anthocyanin with different reagents: pure compounds (quercetin and caffeic acid) and extracts of grape sections (seed, Semillon skin, Semillon pulp).

Certain extracts produced a decrease in color suggesting that competing equilibria are important in the copigmentation effect and that an "anti-copigmentation" effect is possible. Flavonols appear to be the best copigmentation cofactors, and the concentration of quercetin-3-O-glucoside was found to correlate with the strength of copigmentation. Flavonols appear capable of displacing cofactors derived from seed extracts.

Keywords: grapes; wine; copigmentation; color; anthocyanins

1. Introduction

Color is an important factor in the evaluation of red wine quality (Mazza, 1995). Wine color is related to the accumulation of anthocyanins in the grape berries and particularly the skin where five different anthocyanidins are present: delphinidin, cyanidin, petunidin, peonidin and malvidin (Mazza, 1995). In *Vitis vinifera* these typically exist in the 3-monoglucosidic form, while in other *Vitis* species 3,5-diglucosidic anthocyanins are also encountered (Kennedy et al., 2006). The anthocyanins can be esterified by p-coumaric or acetic acid and, to a lesser extent, by caffeic acid (Kennedy et al., 2006). Anthocyanins can exist in several equilibrium forms in wine, with only the flavylium form appearing red in color (aromatic C ring character can only be gained through the structure having a positive charge) (Allen, 1998). Only a small proportion of the anthocyanins in red wine are in the red flavylium form at wine pH (generally less than 10%), with most of the species existing as the hemiacetal product (Kennedy et al., 2006). Much of the chemistry of anthocyanins derives from the interplay between the reactivity of the positive charge and the stability associated with an aromatic ring system in ring C (Allen, 1998).

However, it is not only the anthocyanin content and profile that is responsible for wine color: it is now apparent that copigmentation can account for between 30 and 50% of the color in young wines (Boulton, 2001; Hermosìn-Gutiérrez, 2003). Copigmentation in wine results from molecular interactions between anthocyanic pigments and other organic molecules, called cofactors, forming molecular associations or complexes. The phenomenon has long been recognized in flowers and fruits (Boulton, 2001; Hermosìn Gutiérrez et al., 2005; Robinson & Robinson 1931). The usual cofactors include a variety of compounds such as phenolic acids, flavonoids, and particularly derivatives of the flavonols and flavone subgroups, amino acids and alkaloids and even anthocyanins themselves. Copigmentation generally results in enhanced absorption, a hyperchromic effect, and may also result in a shift in the wavelength of maximum absorption of the anthocyanic pigment to shorter (hypsochromic shift) or longer wavelength (bathochromic shift). A bathochromic shift of 5 to 20 nm typically introduces a blue-purple tone in an otherwise red solution (Boulton,

2001; Levengood et al., 2004; Davies & Mazza, 1993; Gris et al., 2007 ; Castañeda-Ovando et al., 2009).

The complexes can be thought of as either vertically stacked molecular aggregates involving π-π interactions or more perpendicular alignments based on CH-π interactions (Levengood et al., 2004; Liao et al., 1992). Weak molecular forces are involved including hydrophobic interactions in which phenolic entities associate with each other to minimize interactions with water. There are also likely contributions of counter ions such as bitartrate where ionizable cofactors are involved. The copigmentation process increases the proportion of colored forms of the anthocyanins at equilibrium since the stacking of one phenol against another is easier in the case of the colored forms than for the less planar colorless forms (Levengood et al., 2004; Liao et al., 1992). The enhancement in colour due to phenolic cofactors has been found to range from 60-70% for cinnamic acids, up to 1000% with rutin (Bolton, 2001).

In this paper we report the use of spectrophotometry and post-column reaction HPLC to investigate copigmentation in three grape varieties, Pinot Noir, Shiraz and Sangiovese. Pigment sources were skin extracts of each variety, skin plus pulp extract of Shiraz and standard anthocyanins. By using extracts of various grape fractions and standard phenols as cofactors for copigmentation, we aimed to further the understanding of the copigmentation phenomenon in terms of how extracts from the different parts of the fruit interact with extracts of pigments.

2. Materials and Methods

2.1. Chemicals

Anthocyanins (malvidin chloride, malvin chloride), obtained from Extrasynthese (Genay, France) and other phenols (quercetin and caffeic acid), from Sigma (NSW, Australia), were used without further purification. Solvents used were reverse osmosis water (Continental Water Systems Corp. Modulab Analytical Laboratory Research Grade Water System, Seven Hills, NSW, Australia) and AR grade ethanol obtained from Chemsupply (Gillman, SA, Australia).

83

84 *2.2. Grape samples* and extracts

85 Samples of Shiraz, Sangiovese, Pinot Noir and Semillon grapes (approximately 2 kg each) were
86 collected in vineyards in New South Wales (Australia) approximately 2-weeks post-veraison on 12th
87 February 2008, and frozen until analyzed. Shiraz (clone BVRC12) and Semillon (clone DA16162)
88 were sourced from vineyards at Charles Sturt University (Wagga Wagga). They were grown on
89 their own rootstock and they were planted in 2002. Sangiovese (clone H6V9) and Pinot Noir (clone
90 MV6) were collected in a vineyard located at Tumblong. Both were grown on their own rootstock
91 and they were planted in 1998.

92 Sources of anthocyanic pigments were extracts of the skin of Pinot Noir, Shiraz and Sangiovese
93 berries, and standard solutions of anthocyanins. Solutions of standards were prepared by dissolving
94 malvidin chloride (5 mg), malvin or malvidin chloride (10 mg) in 50 ml of model wine base
95 containing 12% ethanol and 2.5 g/l tartaric acid adjusted to pH 3.3 with aqueous NaOH. Pigment
96 solutions of grape skin extracts were prepared by careful removal of the entire skin from 30 berries,
97 and suspension of the skins in 60 ml of the same buffer solution. The mixture was allowed to stand
98 for 6 h with constant shaking after which time the pigment extracts was recovered by filtration.

99 Sources of cofactors were quercetin and caffeic acid standards, seed of the three cultivars
100 (1:1:1), and skin or pulp from Semillon berries (white grapes). Cofactor extracts of standards were
101 prepared by dissolving 3000 mg/l quercetin (0.01 M) or 1788 mg/l caffeic acid (0.01 M) in ethanol.
102 Cofactor extracts of entire seeds (50 g), skins (50 g) and pulp (200 g) were prepared extracting in
103 ethanol (200 ml) by continuous shaking for 24 h in the case of skins and pulp and 40 h for seeds
104 after which time the cofactor extracts were recovered by filtration as above.

105

106 *2.3. Spectrophotometric analysis*

107 A Varian Cary 50 UV-Vis spectrophotometer (Mulgrave, Vic, Australia) was used to record
108 absorption spectra between 190 and 800 nm. The spectra of pigment extracts were measured as

prepared and at dilutions 1:3, 1:5 and 1:10 with cofactor extracts or ethanol as control. The analyses were performed in triplicate.

2.4. HPLC analysis

Analyses were performed on a Varian Prostar 240I HPLC (Mulgrave, Vic, Australia) using a Phenomenex Gemini 5 µm C18 110A 250 x 4.6 mm column (Lane Cove, NSW, Australia), with a Phenomenex SecurityGuard column, operated at a temperature of 25°C. *Gradient elution*: The method of Kammerer et al. (2004) was used for characterization of pigment and cofactor extracts. This involved gradient elution with a mobile phase comprising water:formic acid:acetonitrile (87:10:3 v/v/v, eluent A; 40:10:50 v/v/v, eluent B) and a gradient program as follows: from 10 to 25% B (10 min), from 25 to 31% B (5 min), from 31 to 40% B (5 min), from 40 to 50% B (10 min), from 50 to 100% B (10 min) from 100 to 10% B (5 min). The flow rate was 0.8 ml/min. The eluent was monitored by a Varian Prostar 335 photodiode array detector (Mulgrave, Vic, Australia) at 520 nm for anthocyanins and 280 nm for other phenols. *Modified gradient elution*: This was used in conjunction with post-column reaction to ensure that all anthocyanins eluted in an isocratic region to avoid solvent induced spectral changes. The gradient program was as follows: from 10 to 40% B (5 min), isocratic elution at 40% B (21 min), from 40% to 100% B (6 min), from 100 to 10% B (3 min) with an equilibration time of 5 min. In instances where the concentration of acylated pigments is very low or undetectable, e.g. Pinot Noir and Sangiovese, the isocratic period can be reduced from 21 min to 7 min.

2.5. LC-MS

LC-MS was performed on a Micromass Quattro micro tandem quadrupole mass spectrometer (Waters, Manchester, UK). LC separation was provided by a Waters liquid chromatograph (Milford, USA), consisting of a 2695 Separation Module and 2487 dual wavelength UV detector operated at 520 and 280 nm using the same conditions as for HPLC except for modification of the

mobile phase to reduce the percentage of formic acid which interfered with the mass spectral detection. For this reason, the mobile phase of the normal elution was modified as water:formic acid:acetonitrile (96:1:3 v/v/v, eluent A; 44:1:55 v/v/v, eluent B). Data were acquired by the Masslynx data system for both the MS and UV data. The mass spectral data were acquired for four alternative scans; Scan 1: Positive ion mode, cone voltage 35 V; Scan 2: Positive ion mode, cone voltage 70 V; Scan 3: Negative ion mode, cone voltage 30 V; Scan 4: Negative ion mode, cone voltage 70 V. All scans were performed in the range 80 to 1500 m/z in 1 sec.

2.6. Post Column Reaction parameters

Red grape pigment extract (25 µl) was injected using a Varian Prostar 410 Autosampler (Mulgrave, Vic, Australia) and separation was performed as described for *Gradient Elution* and monitored by a Varian Prostar 335 photodiode array detector (Mulgrave, Vic, Australi a). In the post column reaction method, the column effluent was mixed via a valve and a 1.4 m loop with 5 M NaOH provided at a flow rate of 0.1 ml/min from a second HPLC pump (Waters Model 600E Multisolvent Delivery System). This adjusted the solution pH (1.62) to a pH more closely approximating that of grape and wine (pH 3.3). Cofactor extracts or ethanol as control added via a third HPLC pump (Varian Prostar 210 Solvent Delivery Module) at a flow rate of 0.3 ml/min using a valve and an 8 m reaction coil. The ratio between the flow rates determined the pigment/cofactor ratio: 25 µl of samples with flow rate of 0.8 ml/min in the first pump, diluted with 0.1 ml/min of NaOH 5M, were added by 0.3 ml/min of cofactor solutions or pure ethanol. The effluent was then passed through a Prostar 335 photodiode array detector (Mulgrave, Vic, Australia) to monitor any spectral changes.

3. Results and Discussion

3.1. Characterization of extracts

Pigment extracts of the three grape varieties were profiled by HPLC at 520 nm (Fig. 1a) to characterize anthocyanins. The chromatograms show that the anthocyanin profile of the varieties

differed in the nature and amount of phenols and particularly in the B ring substitutions and also in the extent of acylation. In all varieties, malvidin-3-O-glucoside was the major anthocyanin with lesser amounts of peonidin, petunidin, cyanidin and delphinidin derivatives (-3-O-glucoside and their corresponding acetic and p-coumaric esters). Identification was confirmed by LC-MS. Shiraz exhibited a typical grape anthocyanin profile with a high content of malvidin derivatives (ca. 70% calculated as percentage of total peak area). Pinot Noir had a high amount of both malvidin (ca. 65%) and peonidin (ca. 25%) derivatives while Sangiovese had ca. 50% derivatives of malvidin but with the remaining part of the profile distributed in equal amounts among the other pigments. The profile of Pinot Noir showed no acylated pigments and Shiraz had a high content of acylated anthocyanins (ca. 22% of acetyl-anthocyanins and 18% of p-coumaroyl pigments). Concerning Sangiovese, this grape variety usually accumulate small amounts of these compounds (1-10% of the total pigments content) (Rustioni et al., 2011a). In our samples, this percentage was reduced, probably due to the Australian climate, as the solar exposure decrease the relative content in acylated anthocyanins (Downey et al. 2004, Rustioni et al., 2011b; Haselgrove et al., 2000).

All extracts were profiled by HPLC at 280 nm, which is a more characteristic absorption band for phenols. Representative chromatograms of the pigment extracts are shown in Fig. 1b. At this detection wavelength, anthocyanins appear at 8 – 14 mins and acylated anthocyanins at 22 – 27 mins. At 15 – 20 min the non-pigmented phenols appear. The dominant non-pigmented phenols were flavonols and the major ones in the extracts of the skins from the three red grape varieties were quercetin 3-O-glucuronide, kaempferol 3-O-glucoside and quercetin-3-O-glucoside identified from retention, UV-Vis and mass spectral data (Fig. 1b). The greatest difference between the three varieties in 15 – 20 min region of the 280 nm chromatograms was in the concentration of quercetin-3-O-glucoside (19.5 min). This compound was present in all pigments extracts but at significantly different concentrations: Shiraz, relative concentration 100; Sangiovese, 45 and Pinot Noir, 7.

From HPLC profiling at 280 nm and LC-MS chromatograms of cofactor extracts, flavanols were the major phenolics in the seed extract. In the Semillon skin extract the main phenolics were

187 flavonols. The Semillon pulp extract required a pre-concentration step prior to LC-MS for detection
188 and was characterized by a prevalence of compounds with an odd number molecular mass
189 suggesting the presence of nitrogen-containing compounds rather than phenols.
190
191 *3.2. Preliminary Copigmentation Study*
192 In a preliminary study the effect of seed extract on wine color was examined using a pulp and
193 skin extract from Shiraz grapes. Shiraz berries were sourced from vineyards in Canberra and
194 Griffith from the previous year's vintage. A seed extract and a pulp and skin extract was prepared
195 from these berries using the method described in Materials and Methods. Ethanol concentration was
196 standardized for all spectrophotometric experiments and in the preliminary study this was 24% v/v
197 to facilitate phenolic extraction from seeds. Subsequently, the ethanol concentration was reduced to
198 12%, which more closely resembles that of wine, and ethanol was used as a control to account for
199 changes in spectra due to dilution of pigment extracts. Standardization of ethanol was important
200 because this is a known factor influencing copigmentation reactions (Hermosìn-Gutiérrez, 2003).
201 The addition of seed extract produced a hypochromic effect at the absorption maximum (520 nm)
202 and in fact reduced absorbance at all wavelengths 400-650 nm (Fig. 2). This outcome was
203 unexpected as tannins, which are major phenolic components of seeds, were expected to copigment
204 with anthocyanins and produce a hyperchromic effect (Robinson & Robinson, 1931; Kennedy &
205 Hayasaka, 2004; Es-Safi & Cheynier, 2004; Mirabell et al., 1999; Saucier et al., 2004; Liao et al.,
206 1992). However, our results are consistent with those of Boulton (2001) and we conclude that the
207 seed components (as extracted under our conditions) interfered with the existing copigmentation
208 equilibria in the skin + pulp extract, in wine-like conditions.
209 In order to further explore the copigmentation equilibria in the skin + pulp extract, we
210 separately added quercetin and caffeic acid as known strong cofactors (Boulton, 2001, Baranac et
211 al., 1996, Baranac et al., 1997; Alluis & Dangles, 2001; Hermosìn Gutiérrez et al., 2005;
212 Oszmiański et al., 2004). The addition of quercetin produced both hyperchromic and bathochromic

effects, as expected, depending on relative proportions of the extracts (data not presented). Whereas caffeic acid actually induced a slight hypochromic effect rather than the anticipated hyperchromic effect (Gris et al., 2007, Darias-Martìn et al., 2001; Darias-Martìn et al., 2002; Dimitrić Marković et al., 2000; Davies & Mazza 1993).

From these preliminary data we suggest that the copigmentation equilibrium proposed by Darias-Martìn et al. (2001):

Free anthocyanins + Copigmentation cofactors ⇔ Copigmented anthocyanins

can be re-formulated in the case of grape extracts as:

Anthocyanin + CofactorsA ⇔ Anthocyanin-CofactorsA

Anthocyanin-CofactorsA + CofactorsB ⇔ Anthocyanin-CofactorsB + CofactorsA

Where CofactorsA are derived from skin and/or pulp and CofactorsB are derived from the seeds. This is an example of typical competitive equilibria (Boulton, 2001) where the concentrations of species, plus binding strength will determine the position of the equilibrium and the observed colour. In the original skin+pulp extract, the equilibrium favoured the establishment of the Anthocyanin-CofactorsA complex. However, when the seed extract was added, compounds present in this extract (CofactorsB) disrupted the first equilibrium resulting in the observed hypochromic shifts. Since the loss in colour was more than could be explained by simple dilution, we define this as an "anti-copigmentation" effect. Such an effect has important consequences for winemakers. Some winemaking practices suggest longer extraction times to promote higher concentrations of anthocyanins in the must and hence greater colour. However, this will also result in more seed phenols being extracted, which may negate any colour enhancement that may have been expected. The potential for phenolic extracts from seeds to reduce wine quality has been recently proposed by Ristic et al. (2010)

3.3. Spectrophotometric analysis of copigmentation

Other varieties of grapes were sourced from different regions in order to further explore co- and anti-copigmentation effects. Three were chosen as described above with different anthocyanin profiles and different levels of flavonols, and hence different inherent abilities to form copigments. The pigment extracts of the three varieties showed notable differences in the observed color, with Shiraz producing much darker and more blue extracts, while Pinot Noir extracts were more pale, and Sangiovese intermediate. These differences were reflected in the subtle differences in the UV-Vis absorption spectra, even though each extract had a similar absorbance value at 520 nm. Absorbance spectra of the individual anthocyanins comprising the skin extracts range from 516 nm (cyanidin) to 532 nm (p-coumaric acid ester of malvidin). However the phenolic profiles of the pigment extracts (as above) do not support a simple interpretation of the relationship between colour and anthocyanin content. We therefore conclude that copigmentation plays a fundamental role in determining the color in these extracts (Boulton, 2001).

Pigment extracts of the skin of Pinot Noir, Shiraz and Sangiovese berries and a skin-pulp extract of Shiraz berries were diluted (1:3, 1:5 and 1:10) with solutions of the various potential cofactors (quercetin, caffeic acid, seed extract of the three cultivars (1:1:1), and an extract of the skin or pulp from Semillon berries) using ethanol dilution as a control. The only consistent effects of the various additions were that quercetin always produced a bathochromic shift and, in the case of Pinot Noir, the control always produced the lowest absorbance in the region of interest (510 – 560 nm). With extracts of Shiraz and Sangiovese, the control showed the highest absorbance in a number of instances. For example at a 1:3 dilution of the Shiraz pigment extracts the control is always the highest absorbing solution, whereas the Sangiovese pigment extract (1:5 dilution) shows the control with a mid-range absorbance. The variable responses of the pigment extracts to cofactor additions may be understood in terms of the modified copigmentation equilibria proposed above. As the control is consistently highest in the Shiraz pigment extract, the anthocyanin/CofactorsA equilibrium lies predominantly to the right, such that addition of cofactor extracts (i.e. CofactorsB) does little to disrupt this equilibrium. At the other extreme, the anthocyanin/CofactorsA equilibrium

for Pinot Noir appears weak and any additional cofactor extract increases the level of copigmentation. Sangiovese lies between these two extremes. The importance of copigmentation in Syrah/Shiraz wine has already been demonstrated (Hermosìn Gutiérrez et al., 2005).

The spectrophotometric results are also consistent with the flavonol content of the different varieties (above). Shiraz skin extract – having the highest levels of flavanols also had the highest levels of copigmentation and *vice-versa* for Pinot Noir skin extract. Previous work has highlighted the importance of flavonols as cofactors for copigmentation (Schwarz et al., 2005; Hermosìn Gutiérrez et al., 2005; Mirabell et al., 1999, Boulton, 2001; Asen et al., 1972; Liao et al., 1992; Downey et al., 2003).

Solutions of pure pigments (malvidin chloride and malvin chloride) were prepared using the same solvent as in the extraction of the red grape skins. On addition of cofactors, the largest enhancement in absorbance was observed for quercetin, with much less pronounced absorbance increases occurring with additions of caffeic acid, skin, seed or pulp extract. The differences in results between the pure pigments and pigment extracts (above) suggest that the copigments initially formed in solution from extraction of the grape skins are complex. Simply modeling the interactions with the most abundant anthocyanin in red grape skin, malvidin chloride, did not produce results consistent with those obtained with skin extracts. Further work is required to understand the initial copigments present in grape skin extracts to elucidate the critical pigment co-factor interactions.

3.4 Post-Column Reaction

As mentioned above, spectrophotometric results with pure compounds malvidin chloride and malvin chloride did not provide much insight as to the copigmentation phenomena observed in skin pigment extracts. A possible means to further investigate copigmentation between individual anthocyanins and the cofactor extracts is through a post-column reaction system (Koleva et al., 2000). The system developed for this study is more elaborate than those previously reported due to

291 the fact that the eluent from the column required a pH adjustment prior to reaction with cofactor
292 extracts. Thus three pumps were required: 1 to deliver mobile phase to the column, one to deliver 5
293 M NaOH to adjust the pH, and the third to deliver the cofactor extracts. The advantage of this
294 system is that all detectable anthocyanins can be individually reacted with cofactor extracts without
295 having to first separate them (e.g. by preparative HPLC) or purchase them individually. On the
296 other hand, such a system, with three separate pumps delivering various reagents was technically
297 challenging to construct and there was a large increase in system pressure possibly due to
298 temperature increase caused by the reactivity of the reagents in the mixing coils (especially 5 M
299 NaOH for pH adjustment). The large increase in system pressure also prohibited the use of two
300 detectors, which normally operate in a post-column set-up, one to monitor the eluent from the
301 column and the second to monitor the reaction products. This necessitated two runs to monitor
302 changes to the chromatograms due to reactions with the cofactor extracts. Furthermore, because
303 copigmentation is dependent on solvent composition, a new elution profile, where the anthocyanins
304 are eluted isocratically, had to be developed. Additional factors to consider when using a post-
305 column reaction system are that the short mixing time may not allow enough time for the
306 cofactor/pigment equilibria to be fully established, and the solvent composition (especially the
307 presence of acetonitrile) may not be most conducive to a maximal copigmentation effect. Both
308 factors may reduce the overall extent of copigmentation, but the results we have obtained show that
309 the system was reproducible (see below).
310 A consequence of the new elution conditions was that some co-elution of anthocyanins and other
311 phenolic compounds occurred. For example, under isocratic conditions quercetin-3-O-glucoside and
312 peonidin acetate co-elute (although not completely overlap). The visible absorbance spectrum,
313 generated from the PDA detector (Fig.3), shows that the spectrum for peonidin acetate (in the
314 region of overlap with quercetin-3-O-glucoside) is not the same as for peonidin acetate in the
315 gradient elution chromatogram, where peonidin acetate is separated from quercetin-3-O-glucoside.
316 In particular, a hyperchromic effect (ca. 15% increase in absorbance) and a bathochromic shift (2.7

nm) were observed (Fig. 3). The observation of this copigmentation effect for quercetin-3-O-glucoside is consistent with earlier results in that Shiraz was identified as having the highest amounts of quercetin-3-O-glucoside and also the highest level of copigmentation. Likewise, Pinot Noir had the lowest amounts of quercetin-3-O-glucoside and the lowest level of copigmentation.

The post-column reaction analysis was performed using the various cofactors with the exception of the Semillon skin extract, which when trialed resulted in detrimental increases in back pressure in the chromatographic system. The most interesting effect was observed for the seed extract although all reagents had some copigmentation effect. The hyperchromic effect of the seed extract was most noticeable in producing a broad region of raised baseline intensity between 4.5 min and 11 min (Fig. 4a). This effect was common for skin pigment extracts from all three cultivars. The additional color, which appears during the elution of the non-acylated anthocyanins and reaction with the seed extract, may have significance for the color of red wine. Specifically, it highlights a potential role for compounds derived from seeds in influencing wine color.

The region of raised baseline intensity comes to an end near the retention time of the acylated anthocyanins (beginning at ~11.5 min, Fig. 4a). This region of the chromatogram also coincides with the elution of flavonols. One possible explanation for the lack of baseline intensity in this region is an interference of the flavonols in the interactions made among proanthocyanidins (tannins, derived from seeds) and acylated anthocyanins (derived from skins). This would be consistent with the observation of Oszmiański et al. (2004) who found that flavonols formed more stable copigments with acylated anthocyanins than with non-acylated anthocyanins. In fact, in our isocratic method peonidin acetate co-eluted with quercetin-3-O-glucoside and other flavonols and the baseline became flat at the retention time of these compounds.

The post column reaction system showed very good reproducibility (±0.1 nm) in the evaluation of a bathochromic effect. The strongest bathochromic reagent was quercetin, with an average increase of maximum absorption wavelength of 1.7 nm over the three red grape cultivars. Considering the non-acylated anthocyanins, the effect is higher with an increase of the substitution degree and the methyl

343 substitution (Table 1). It has been established that the substitution pattern of the phenolic B ring and
344 the acylation of the pigments affect copigmentation. Davies and Mazza (1993) concluded that the
345 number of oxygen substituents and the presence of a methyl group has a greater magnitude shift
346 than the presence of diacylation. Our data confirm this relationship.
347 The effect of the pulp extract was interesting in that it produced a hypsochromic change. This may
348 explain the difference between berry color (blue-purple) and the more reddish tones in the finished
349 wine.
350

351 ## 3. Conclusion

352 Mirabel et al. (1999) stated some cofactors "were found as "good" copigments – rutin, quercetin
353 (flavonols) and proanthocyanidins … – and others acting as "bad" ones – (-) epicatechin, (+)
354 catechin". In this paper, we have examined what happens in a complex sample where both "good"
355 and "bad" cofactors are present in both the extracts of grape skin and other extracts, pulp, seed etc.
356 Such complexity is more like that found when grapes are crushed and during fermentation where
357 pigments and cofactors interact with one another in competing equilibria. During the course of this
358 investigation, we discovered that with certain cofactors, the equilibria can be forced in a direction to
359 yield a decrease in color intensity. This anti-copigmentation effect may be of importance to
360 winemakers as it shows that a decrease in color may result from certain combinations of phenolic
361 compounds especially those from seeds.
362 The results underline the fundamental role of flavonols, and their importance in wine color suggests
363 some technical consequences. Because of their function as UV screening agents, their biosynthesis
364 can be managed in vineyards by leaf removal and bunch exposure (Downey et al., 2004; Ryan et al.,
365 1998; Kolb et al., 2003; Haselgrove et al., 2000; Ristic et al., 2007; Tarara et al., 2008). While they
366 can be useful in young wines, enhancing their color as strong cofactors, their potential to interfere in
367 color stabilization by breaking tannin-anthocyanin interactions must also be considered.
368

ACKNOWLEDGEMENT

The authors thank Dr Bruno Holzapfel for donation of grape samples, Mr. Michael Loughlin for assistance with the post-column reaction assembly, and Dr Daniel Jardine for acquiring the LC-MS data. We acknowledge Charles Sturt University and the University of Milan for financial support for this work.

Figure Captions

Fig. 1. Skin extracts characterization HPLC chromatograms. a1: Shiraz 520 nm, a2: Sangiovese 520 nm, a3: Pinot Noir 520 nm; (peak correspondence = a: malvidin-3-O-glucoside, b: peonidin-3-O-glucoside, c: petunidin-3-O-glucoside, d: delphinidin-3-O-glucoside, e: cyanidin-3-O-glucoside, f: acetic ester of malvidin-3-O-glucoside, g: p-coumaric ester of malvidin-3-O-glucoside, h: acetic ester of peonidin-3-O-glucoside, i: p-coumaric ester of peonidin-3-O-glucoside); b1: Shiraz 280 nm (peak correspondence = l: caffeic ester of malvidin-3-O-glucoside, m: quercetin-3-O-glucoside), b2: Sangiovese 280 nm, b3: Pinot Noir 280 nm.

Fig. 2. Visible spectrum of extracts of Shiraz grapes. "Berry" extract comprised seed+pulp+skin

Fig. 3. Comparison of the spectra of peonidin acetate extracted from HPLC chromatograms under gradient elution ("normal") and isocratic conditions. (a) Spectra normalised to 100% to show the bathochromic shift due to co-elution of quercetin-3-O-glucoside. (b) Full scale spectra showing hyperchromic effect of co-eluting quercetin-3-O-glucoside.

Fig. 4. HPLC chromatograms of the post column reaction system monitored at 520 nm. (a) Chromatogram obtained from post column addition of seed extract showing raised baseline from 4.5-11 min. (b) Chromatogram of control system, i.e. ethanol added post column

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493

Table 1.

Wavelength shift (nm) in the wavelength of maximum absorption for different pigments in the extract of red grape skin obtained in the Post Column Reaction analysis

Pigment	**Seed**	**Quercetin**	**Caffeic acid**	**Pulp**
Delphinidin	0.5	1.4	0.3	-3.9
Cyanidin	0.5	1.1	0.1	-3.4
Petunidin	0.3	1.9	0.2	-3.5
Peonidin	0.5	1.5	0.2	-3.0
Malvidin	0.2	2.3	0.0	-3.3
Malvidin acetate	-0.1	1.8	0.1	-3.1
Malvidin p-coumarate	0.4	1.8	0.1	-2.4
Average	0.3	1.7	0.1	-3.2

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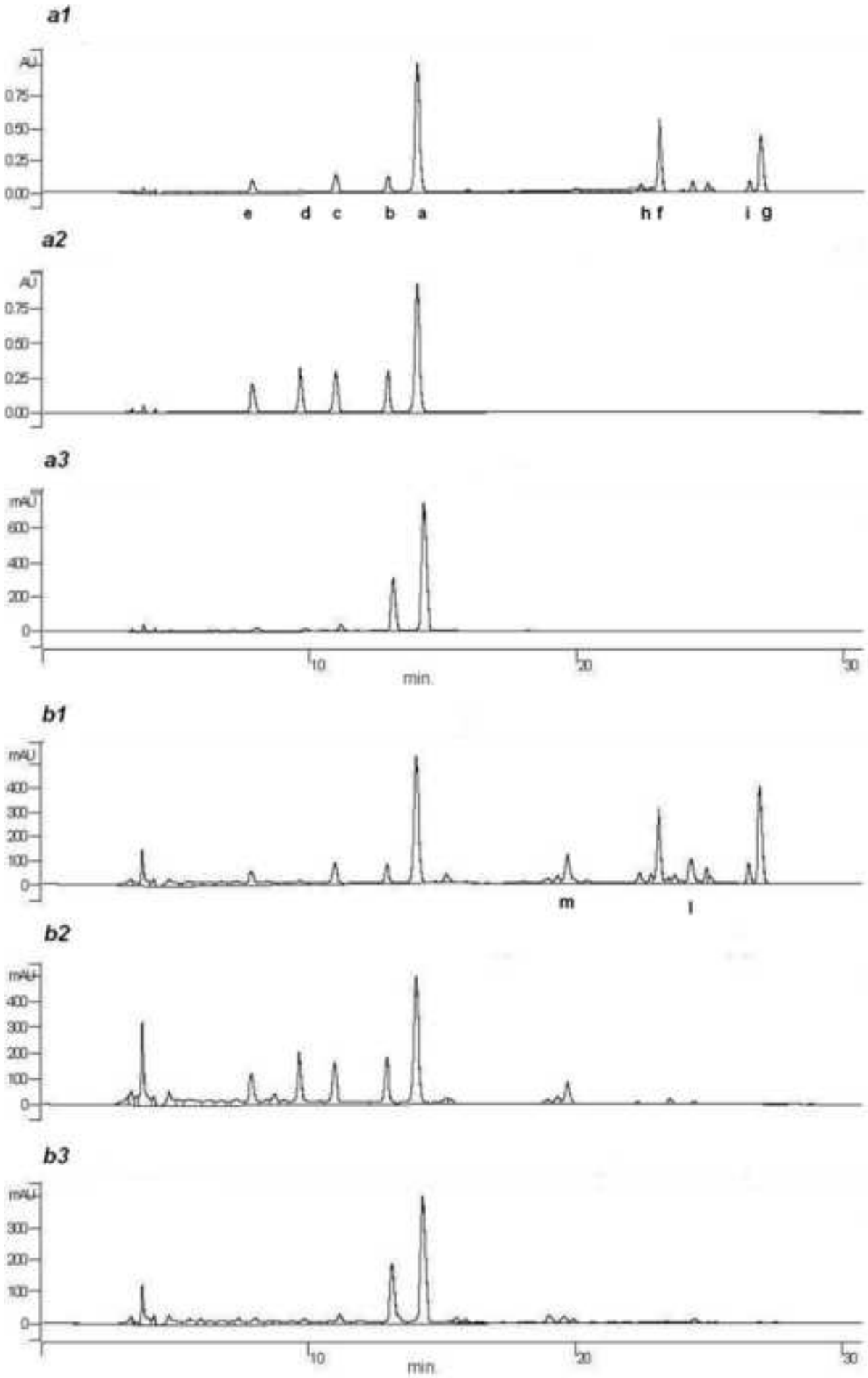

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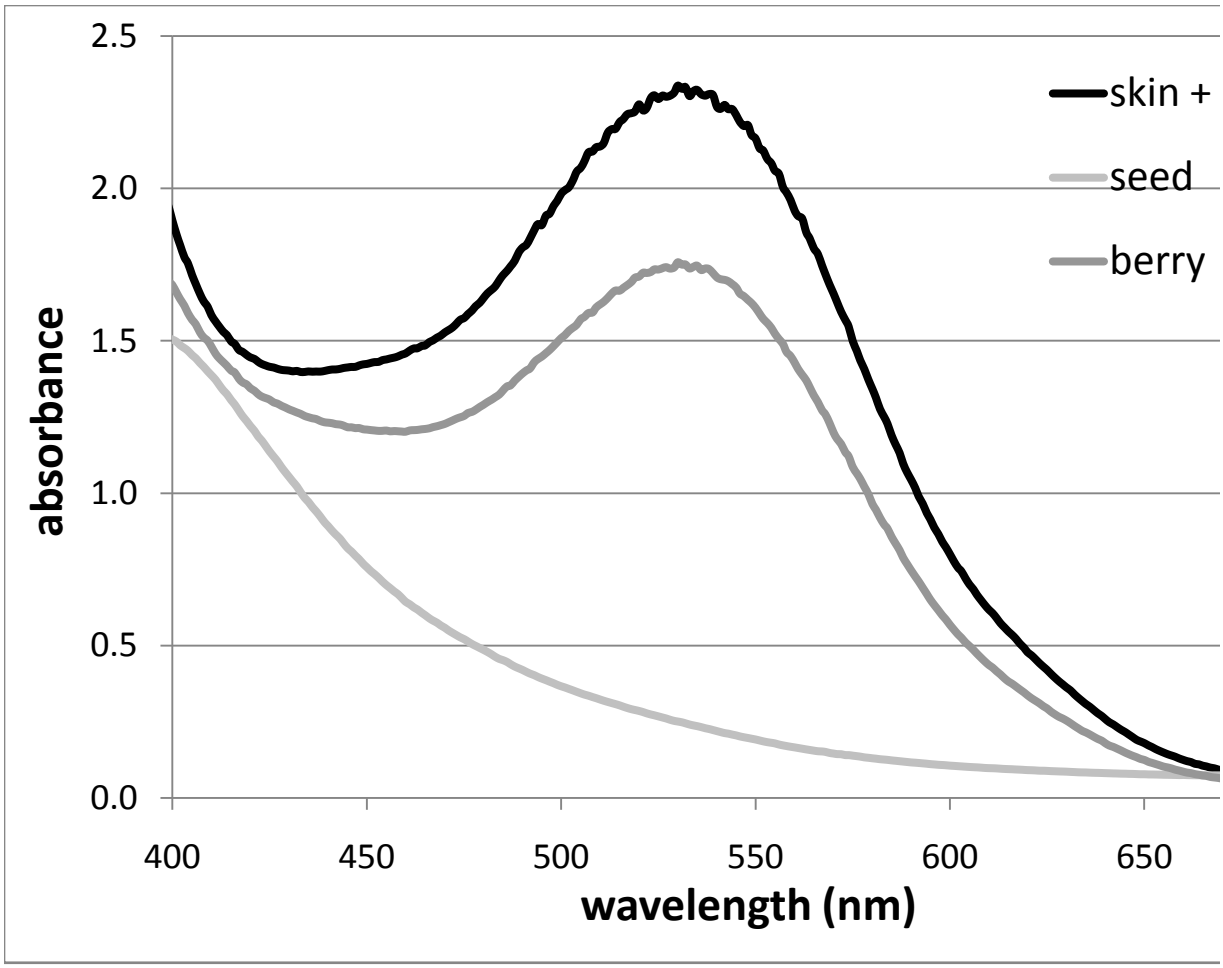
2.5
2.0
1.5
1.0
0.5
0.0
absorbance
400
450
500
550
600
650
wavelength (nm)
skin +
seed
berry

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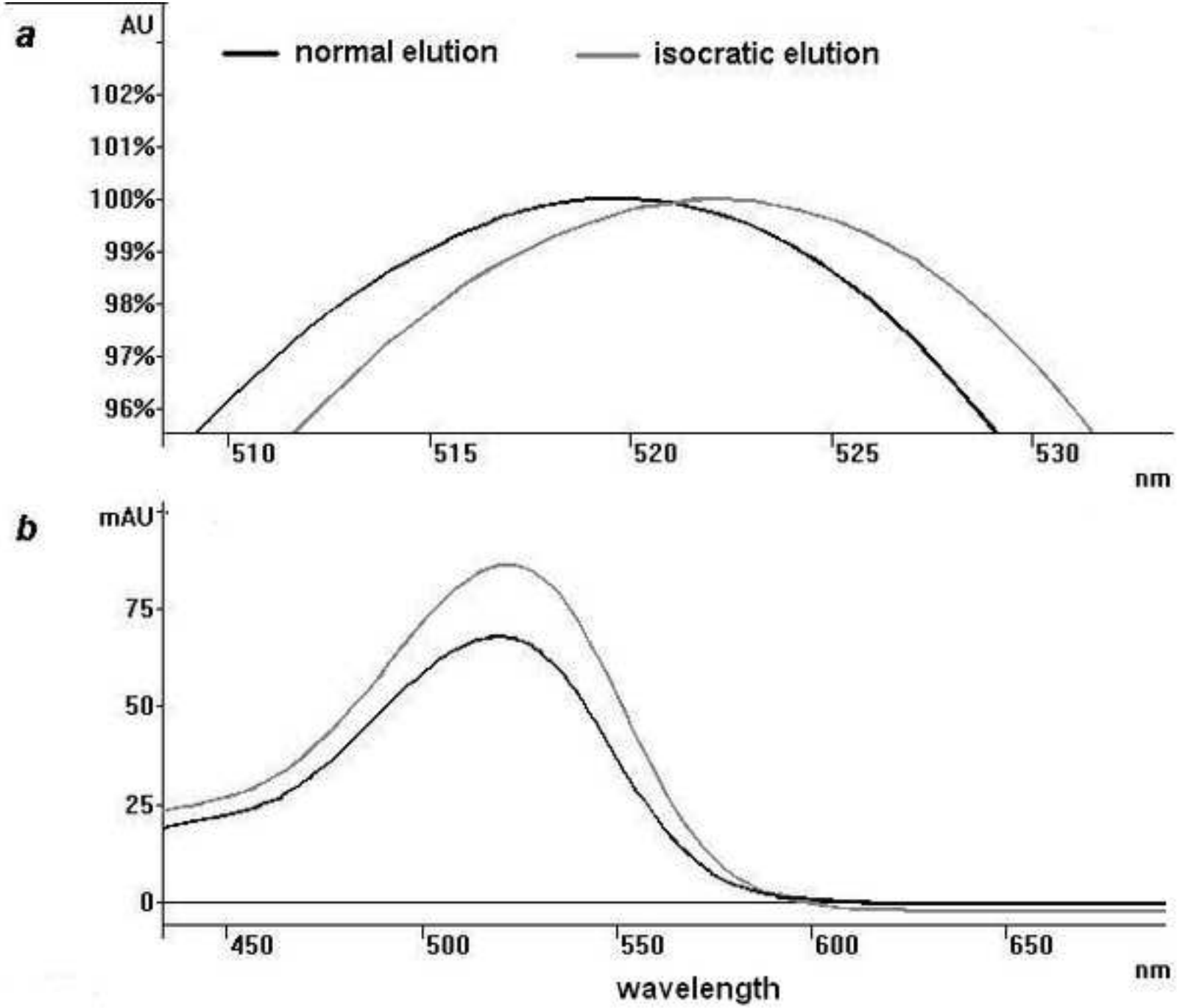

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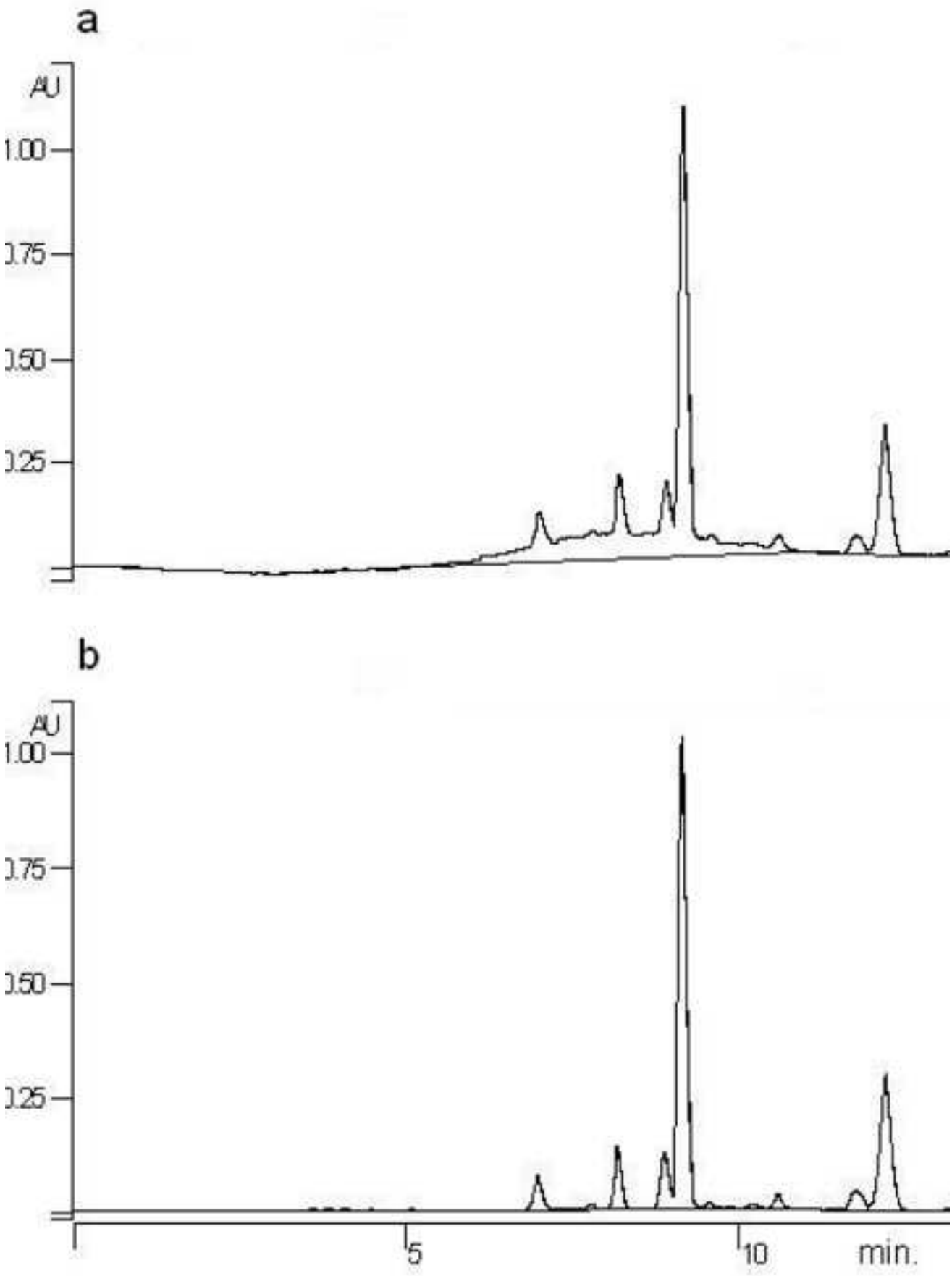

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