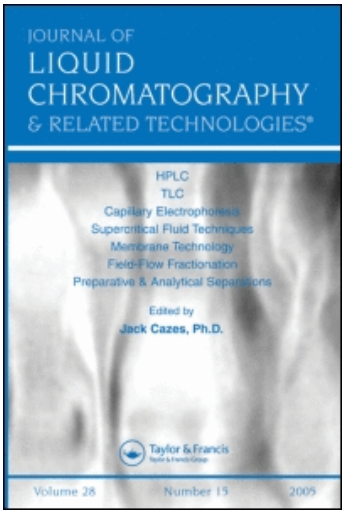

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METHOD-DEPENDENT BIAS IN THE QUANTITATION OF *CIS*- AND *TRANS*-RESVERATROL GLUCOSIDES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The concentrations of *cis* and *trans*-polydatin (resveratrol 3-β-glucoside) were measured in 133 red wines by two methods: Method A utilized normal phase HPLC with isocratic elution followed by UV detection, and Method B was based on reverse phase HPLC with gradient elution and photodiode array detection. In each method, 20 μL of sample was directly injected without prior treatment.

For both polydatin isomers, Method B gave higher values. These discrepancies were more marked with the second of two Calibrators prepared by purification of *trans*-polydatin from the dried roots of *Polygonum cuspidatum*. Overall, the correlation coefficients for the two methods were only 0.442 and 0.704 for the *cis* and *trans* isomers, respectively. No explanation for these findings was forthcoming. These results demonstrate the need for caution in interpreting and comparing values for polydatins reported using different methods, and emphasize the need for pure synthetic standards of these compounds to allow valid inter-laboratory and inter-method comparisons.

INTRODUCTION

Widespread interest in the polyphenolic constituents of wine driven, at least in part, by recognition of their health-promoting properties,[1] has generated an epidemic of publications using a diversity of techniques, although GC-MS and HPLC methods have been the most popular. Rarely have different methods for the same constituent been compared, although a scrutiny of literature values for wine polyphenols reported by different investigators reveals marked discrepancies highly suggestive of method bias that demands exploration and explanation. In one of the few investigations along these lines,[2] we compared four methods for the assay of resveratrol isomers and observed systematic differences, some of which could be explained by technical anomalies such as *trans*-to-*cis* isomerisation, and thermal degradation of resveratrol glucosides. We now present a comparison of data for *cis* and *trans*-polydatin (resveratrol 3-β-glucoside) obtained by analyzing the same red wine samples using two HPLC methods, and two standards for the polydatin isomers, both purified from the dried roots of *Polygonum cuspidatum* as previously described.[3]

EXPERIMENTAL

Normal Phase HPLC (Method A)[4]

A ternary HPLC (Spectra-Physics, San Jose, CA, USA) coupled to an SP 8000 pump, SP 8875 autosampler and Chrom-Jet integrator was used with a LiChrospher 100 CN (5μm) column (250 x 4 mm I.D. Merck, Darmstadt, Germany) in the normal-phase mode. The mobile phase was water-acetonitrile-methanol (90:5:5, v/v) and the flow-rate (isocratic) was 1 mL/min. Untreated wine (20 μL) was directly injected and absorbance monitored at 306 nm using a 2550 variable-wavelength UV detector (Varian Instruments, Mississauga, Ont.,

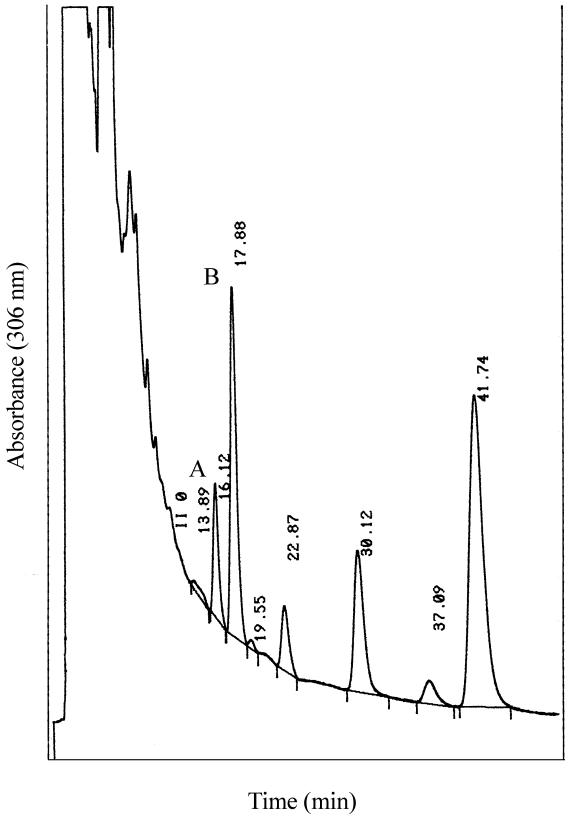

Figure 1. Typical chromatographic separation of *cis*-polydatin (A) and *trans*-polydatin (B) employing Method A.

Canada). A typical separation is shown in Figure 1. A four-point calibration curve based on peak area was run at the beginning of each day, and a standard was injected after every tenth sample. Provided that the nearest standard was within ± 8% of the appropriate absorbance on the calibration curve (the CV at these concentrations was 3.9%), the polydatin concentrations of the unknowns were read directly from the curve.

On the rare occasions when this criterion was not met, a new calibration curve was prepared and the requisite samples were re-run. c*is*-Polydatin eluted at around 16.1 min and *trans*-polydatin at approximately 17.9 min, the two peaks being well separated (Figure 1).

Table 1

Analytical Characteristics of Methods Used in this Investigation[a]

	Method A	Method B
Linear Range (mg/L)		
cis-polydatin	0.22 - 15.79	1.4 - 10.2
trans-polydatin	0.13 - 8.20	0.9 - 6.8
Recovery (%)		
cis-polydatin	106	94.3 - 101.1
trans-polydatin	102	99.3 - 107.4
Detection Limit (mg/L)		
cis-polydatin	0.20	0.075
trans-polydatin	0.11	0.048
Precision (CV%)		
cis-polydatin	0.4 - 6.7	1.8 - 3.8
trans-polydatin	0.4 - 3.9	10.0 - 2.7

[a] From original publications.[4,5]

The choice of 306 nm as the wavelength of measurement was based upon the fact that this is within 10% of the absorbance maxima of *cis*-polydatin (approximately 295 nm) and *trans*-polydatin (approximately 310 nm) and provides excellent sensitivity for both isomers. The analytical characteristics of both methods are summarized in Table 1.

Reverse Phase HPLC (Method B)[5]

An ODS Hypersil 5 μm column, 250 mm x 4 mm i.d., was used as the stationary phase and was preceded by a guard column of LiChrospher 100 RP-18, 5 μm 4 mm x 4 mm. Both were purchased from Hewlett Packard (Mississauga, ON, Canada). The chromatography equipment, all from Hewlett Packard, comprised the Series 1050 automatic sample injector, solvent degasser, quaternary pump, and diode array detector coupled to the HP Chem-Station utilizing the manufacturer's 2.05 software package.

Samples of 20 μL of wine or calibrator standard were directly injected onto the column and eluted with a gradient comprising acetic acid (pump A), methanol (pump B), and water (pump C). Zero-time conditions were 5% A, 15% B, 80% C at a flow rate of 0.4mL/min. After 5 min the pumps were adjusted to 5% A, 20% B, 75% C at a flow rate of 0.5 mL/min and at 30 min to 5% A, 45% B, 50% C at 0.5 mL/min until termination of the run at 40 min. This was followed by a 10-min equilibrium period with the zero-time solvent mixture prior to injection of the next sample. Detection was routinely accomplished by monitoring the absorbance signal at 306 nm, and peak areas were used to assign the polydatin concentrations of unknown samples by reference to a standard curve based on four dilutions of the calibrator.

Figure 2 shows a typical separation. The software is able to integrate peak areas by curve-regeneration based upon ascending and descending slopes, correcting for high baseline and partial peak overlap. Additionally, Match Factor spectral analysis of each peak assigned a value between 0 and 1000 for concordance between the spectrum of the peak and that of the pure compound. Values above 990 indicate near-identity, and those below 900 suggest that the spectra are different.

Purity checks were also performed at the inflexion points and apex of each peak, and the peak purity plot comprising the three spectra was drawn in a normalized and derivatised mode. Purity Factors > 950 led us to exclude hidden impurities and to consider the peak to be consistent with the presence of a single component.

Standards

Trans-polydatin was purified from the dried root of *Polygonum cuspidatum* according to a published method[3] and a portion was converted to the *cis*-isomer by UV-irradiation.[4] The authenticity of the two purified isomers was validated by HPLC using Method B and UV spectral analysis with the HP Chem-Station 2.05 software package that utilizes both absorbance and derivative spectra to assign Purity and Match Factors as described; these were > 99% for the standards used in this investigation. The concentrations of the two polydatin isomers were assigned by measuring the *cis* and *trans*-resveratrol before and after exhaustive hydrolysis with β-glucosidase using direct-injection GC-MS techniques.[6,7]

Solutions of the two isomers (in methanol) were diluted and mixed to generate Calibrators that had approximately equal concentrations of each. Two Calibrators were employed in this investigation: Calibrator 1 had *trans* and *cis*-polydatin concentrations of 75 mg/L and 63 mg/L, respectively; in Calibrator 2

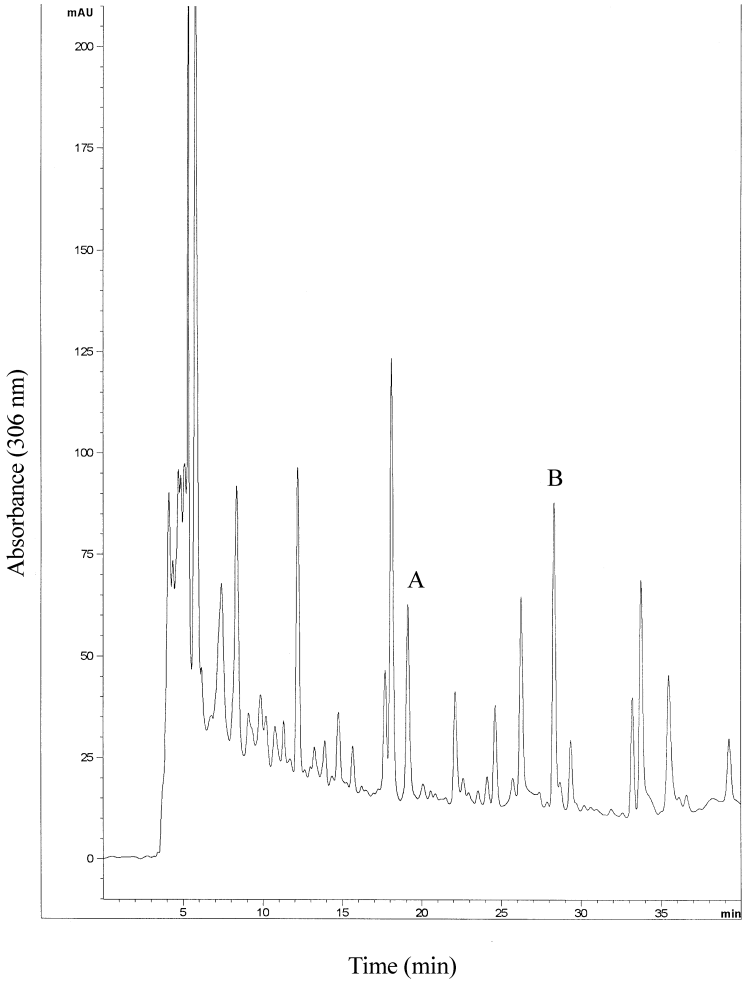

Figure 2. Typical chromatographic separation of *trans*-polydatin (A) and *cis*-polydatin (B) employing Method B.

these concentrations were 80 mg/L and 59 mg/L, respectively. Both were wrapped in foil and stored at –20°C. These values were re-checked by GC-MS analysis every two weeks and the Calibrator was discarded if one isomer showed a fall > 8% of the original value in two successive analyses. Standard curves were prepared by analysing four dilutions of the Calibrators.

Table 2

Concentrations of Polydatin Isomers in Commercial Red Wines Assayed by Two HPLC Methods and Use of Two Calibrators

	***cis*-Polydatin (mg/L)**			**Regression Parameters**	
	Mean	**SD**	**r**	**Slope (SE)**	**Intercept (SE)**
Calibrator 1[a]					
Method A	1.11	0.80	0.492	0.58(0.11)	0.64(0.15)
Method B	1.29	0.95			
Calibrator 2[b]					
Method A	0.64	0.41	0.802	2.21(0.26)	0.26(0.20)
Method B	1.68	1.12			
Both Calibrators[c]					
Method A	0.96	0.73	0.442	0.62(0.11)	0.82(0.13)
Method B	1.42	1.02			
	***trans*-Polydatin (mg/L)**				
Calibrator[d]					
Method A	1.17	0.85	0.683	0.094(0.10)	0.55(0.14)
Method B	1.66	1.16			
Calibrator[e]					
Method A	0.39	0.17	0.296	0.68(0.48)	0.62(0.21)
Method B	0.89	0.39			
Calibrator[f]					
Method A	1.04	0.83	0.704	0.94(0.18)	0.54(0.11)
Method B	1.52	1.11			

[a] n = 90; [b] n = 42; [c] n = 132; [d] n =110; [e] n = 23; [f] n = 133.

Samples

Commercial red wines submitted for routine analysis to the Liquor Control Board of Ontario were used, and both assays were completed within 24 h of opening the bottle.

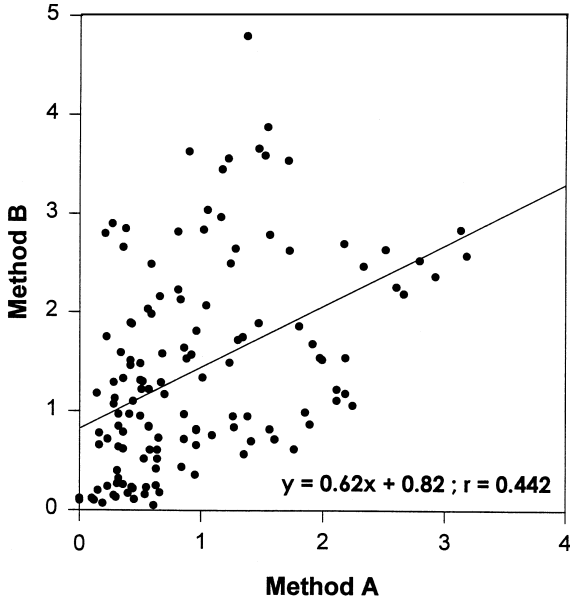

Figure 3. Individual values for *cis*-polydatin concentrations of 132 red wine samples assayed by both methods with both calibrators.

RESULTS

Cis-Polydatin

Using Calibrator 1, Method B gave a mean value for 90 red wine samples 16% higher than that of Method A (Table 2). In 46 samples the latter was higher; in 42 samples the former was higher; and in 2 both gave equal values. Despite this apparently good agreement, the correlation between the two methods was only modest ($r = 0.492$). The intercept and slope of the regression line were significantly different from 0 and 1, respectively ($P < 0.001$ for both).

With Calibrator 2, the mean of 42 samples was 163% higher for Method B than for Method A, the former giving higher concentrations in 39 samples and the latter in 4. The intercept of the regression line was not significantly different from zero, but the slope of 2.21 was highly significant ($P < 0.001$). Despite these discrepancies, much closer correlation between the results of both methods was observed ($r = 0.802$).

When the results for both calibrators were combined (Figure 3), the intercept of the regression line rose to 0.82 ($P < 0.001$) and the slope fell to 0.62 ($P < 0.001$), the correlation between results of two methods declined to 0.442.

Trans-Polydatin

Using Calibrator 1, Method B gave a mean value for 110 wine samples 42% higher than that of Method A (Table 2). In 86 samples, the former was higher; in 22 the latter was higher; and in 2 both gave equal values. The correlation between the two methods was not especially high ($r = 0.683$), but the regression coefficient ($R = 0.94$) was not significantly different from 1.0 although the intercept of the regression line (0.55) was significantly different from zero ($P < 0.001$).

For 23 samples analyzed using Calibrator 2, the mean value given by Method B was 128 % higher than that of Method A and the individual values for the former were higher in 21 of the samples. The parameters of correlation and regression for the two methods were very poor, values for the slope being 0.68 (< 1.0, $P > 0.05$), for the intercept 0.62 (>0, $P < 0.01$) and for the correlation coefficient 0.296 (not significant).

However, when the results for both calibrators were combined, the overall pattern showed some improvement (Figure 4). The slope (0.94) and intercept (0.54) of the regression line were almost identical to those with Calibrator 1, and the correlation coefficient improved to 0.704.

DISCUSSION

We have been unable to identify the reason for the discrepancies between the two methods employed. Both utilized direct automatic injection of 20 μL of wine sample without prior extraction so that variability in recovery does not explain our findings. Method B is the more sensitive of the two (Table 1) and also offers somewhat better precision. However, these factors do not seem to be relevant since imprecision would not introduce systematic bias and sensitivity would affect data only at extremely low concentrations.

The fact that these discrepancies were more marked with the second than with the first calibrator used in this investigation suggests that the material used for standardization may be the principal source of error. In its preparation, we used a published method to purify *trans*-polydatin from a natural source.[3]. The original authors validated their purification by NMR spectroscopy as well as by spectral analysis. We used only the latter.

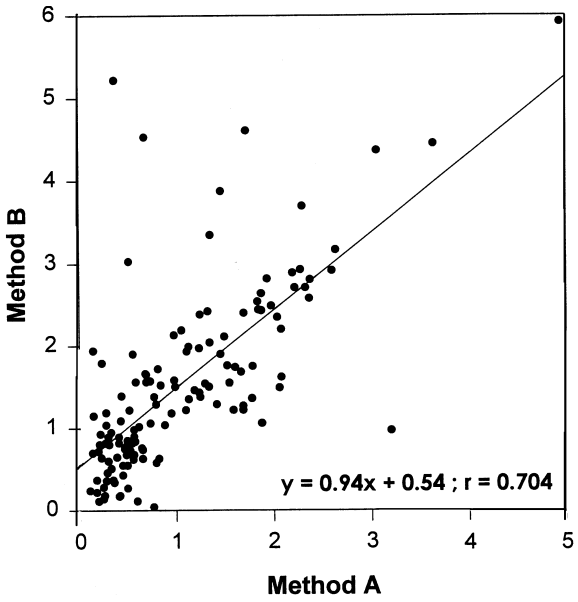

Figure 4. Individual values for *trans*-polydatin concentrations of 133 red wine samples assayed by both methods with both calibrators.

The chromatographic peak corresponding to the eluate selected for standardization, following exhaustive hydrolysis with β-glucosidase, was identical to that of authentic synthetic *trans*-resveratrol and the concentration was assigned after GC-MS analysis.[6] The portion converted to *cis* polydatin by UV irradiation likewise yielded a spectrum perfectly fitting that of *cis*-resveratrol after exhaustive enzymatic digestion with β-glucosidase and its concentration was assigned after analysis by GC-MS.[7]

Re-analysis of the Calibrators formed by mixing the two isomers did not reveal any significant change with time. Nevertheless, it is possible that minor contaminants or matrix effects could have exerted different influences upon the normal and the reverse-phase procedures. It is unlikely that the higher results for Method B were due to contaminants since the software utilized to calculate the concentrations automatically analyses the peaks for spectral purity and concordance with authentic library spectra of the compounds of interest. The requirements for > 95% Purity and > 90% Match Factor were achieved in all except one sample for *cis*-polydatin, accounting for the fact that data for only 132 pairs are presented for this compound. Theoretically, Method A should be more susceptible to falsely elevated values due to unrecognized peak contamination, but this does not seem to be the case.

Table 3

Literature Values for Resveratrol Glucosides in Red Wines of Various Origin (Region or Cultivars)

Origin	Polydatin Concentration (mg/mL) *cis*	*trans*	Analytical Conditions	Ref
Mourvedre		13.4	Reverse phase HPLC; gradient elution; Superspher 100 RP; diode array detection	8
Italy	2.84	0.58 1.16	Reverse phase HPLC; gradient elution; ODS-Hypersil; UV detection	9 10
Spain	1.85	0.76	Reverse phase HPLC; gradient elution; Nuclosil; diode array detection	11
Italy Spain Pinot noir (Burgundy) Pinot noir (Oregon) Canada	2.11 2.40 1.22 1.80 3.35	2.78 3.20 0.94 0.64 2.52	Normal phase HPLC; isocratic elution; LiChrospher 100 CN; UV detection	12
Pinot noir (Slovenia)	2.50	4.10	Reverse phase HPLC; gradient elution; LiChrospher RP; UV detection	13
Canada	1.66	2.06	Solid phase extraction; derivatisation; GC-MS separation and detection	14

Table 3 presents a survey of the published literature on polydatin concentrations in red wines. In some instances, the values are as stated by the authors; in others, visual extrapolation from bar histograms or similar illustrations are presented. Comparing the data for wines of similar origin with respect to region or cultivar, it is clear that there are quite marked differences not only in absolute concentrations but also in the ratios of the two polydatin isomers. While it is to be expected that their concentrations in wines of different vintages could differ quite significantly, one has to recognize the possible contributions of different methods and different standards to this variability.

Within each method, as we noted in detailed comparisons of wines of varying concentrations, the relative order is reasonably consistent, a conclusion validated by a number of parallel assays carried out with GC-MS techniques.[6,7] However, only when pure synthetic *cis* and *trans*-polydatin become available, will it be possible to develop standards that will allow the valid comparison of absolute data from different laboratories or obtained by different methods.

CONCLUSION

A systematic bias between the two methods was identified, superimposed upon which was a further layer of variability attributable to the calibrator employed. This being derived from a natural source and further modified by UV-treatment, may be subject to matrix effects leading to altered dose-response relationships in different chromatographic systems. Commutability of data between laboratories, even when similar methodology is used, may have to await the availability of pure synthetic standards of these glucosides.

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