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Harold Rathburn
Tarleton State University

Peter Bell
Tarleton State University

Scott Cook
Tarleton State University

Darrell D. Mayberry
University of North Texas

Emryse Geye
Portland State University

See next page for additional authors

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A STATISTICAL ANALYSIS OF *TRANS*-RESVERATROL IN GRAPE CANE FROM TEN VARIETIES OF CULTIVATED WINE GRAPES (*VITIS* SPP.)

Harold Rathburn[1*], Peter Bell[2], Scott Cook[3], Darrell D. Mayberry[4], Emryse Geye[5] and Ryann Goodrich[6]

[1]*Department of Biological Sciences, Tarleton State University, Stephenville, TX 76402*
[2]*Department of Chemistry, Geoscience and Physics, Tarleton State University, Stephenville, TX 76402*
[3]*Department of Mathematics, Tarleton State University, Stephenville, TX 76402*
[4]*Department of Chemistry, University of North Texas, Denton, TX 76201*
[5]*Department of Chemistry, Portland State University, Portland, OR 97207*
[6]*Integrity Bio-Chemicals, 1100 N Cresson Hwy, Cresson, TX 76035*
**Corresponding author; Email: rathburn@tarleton.edu*

Abstract.–*trans*-Resveratrol (resveratrol) has been shown to have various health benefits. As a consequence, there is an effort to determine plentiful sources of this molecule. Certain plants, such as grapes, synthesize resveratrol and therefore appear to be an excellent source of this chemical. Annual pruning of grapevine yields significant amounts of cane material that is normally mulched or simply burned. Previous studies have shown this grape cane to contain economically useful resveratrol. Texas is the seventh largest producer of grapes in the USA with over 162 ha currently under cultivation. As a result it is estimated that more than $3.2 million of resveratrol could be extracted from grape canes in Texas each year. In this study resveratrol was isolated by a non-optimized protocol from ten varieties of grape cane grown in central Texas, USA. HPLC analysis showed the cultivars Lenoir and Cabernet Sauvignon yielded the greatest relative amounts of resveratrol (52.3 and 49.6 mg/kgDW). A statistical grouping of the ten varieties suggests that Norton, Blanca du bois, Cabernet Sauvignon, and Lenoir are the best candidates to use for further resveratrol isolation.

Keywords: HPLC, statistical groups, stilbenes, soxhlet extraction

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Stilbenes are a diverse group of economically important chemicals some of which have been used for decades as optical brighteners in soaps, detergents, and fabric softeners (Smulders & Sung 2002). Selected optical brighteners were found to confer UV protection to biological systems (Shapiro & Dougherty 1994) where it indicated that at least a 100-fold reduction of LC_{50} values when an optical brightener was included in solutions of occlusion bodies of various baculoviruses.

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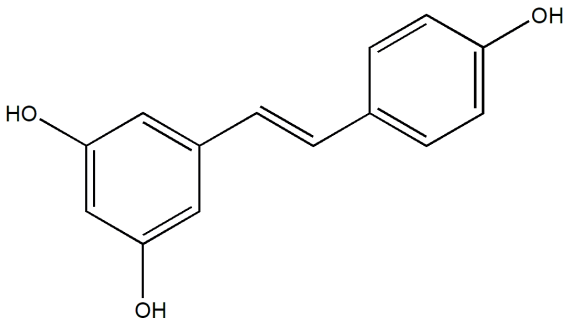

Figure 1. Structure of *trans*-Resveratrol.

Stilbenes are synthesized by biological systems by a well-understood pathway (Kindl 1985) and resveratrol, shown in Figure 1, appears to be the stilbene with the greatest and most varied biological activities most commonly produced by plants. Resveratrol's activities include anti-aging (Haigis & Sinclair 2010), cardioprotection (Hung et al. 2000), prevention of platelet aggregation (Pace-Asciak et al. 1995), inhibition of eicosanoid synthesis (Pace-Asciak et al. 1995) and anticancer agent (Jang et al. 1997), to name a few. The mode of action is still enigmatic, but resveratrol has been shown to bind to low density lipoproteins after consumption of red wine (Urpi-Sarda et al. 2005), inhibit the dioxygenase activity of lipoxygenase (Pinto et al. 1999), and activate SIRT1 (Hubbard et al. 2013).

Resveratrol is synthesized by several plant species, including peanuts, blueberries, grapes and pines (Sotheeswaran & Pasupathy 1993), and can be found in resulting food products, such as wine and peanut butter (Burns et al. 2002). Resveratrol can be found in various tissues of plants. For example, this particular stilbene is found in the stems, leaves, fruit skins, and seeds of grapes (Melzoch et al. 2001; Li et al. 2006). Resveratrol synthesis in grape plants can be induced upon injury, exposure to UV light, or fungal infection (Langcake & Pryce 1976; Romero-Perez et al. 2001).

Resveratrol has been targeted for crop protection because of the aforementioned beneficial traits it possesses. Genes for stilbene

synthesis have been used to transform crops to increase resveratrol content resulting in improved fungal resistance (Chong et al. 2009; Delaunois et al. 2009). Resveratrol can also be applied to fruits post-harvest to potentially reduce fungal infection during storage and shipping (Gonzalez et al. 2003; Montero et al. 2003). As a result, determining sources of ample resveratrol has garnered much interest. Resveratrol can be synthesized utilizing the Heck reaction (Farina et al. 2006), however including a synthetic additive in human food is not favorable. Since resveratrol occurs naturally in several plants of the human diet, more effort has been expended to find a rich plant source of this and other stilbenes. Grapes (*Vitis* spp.) are a popular dietary fruit, as well as for the production of wines, and likely a major contributor of consumed resveratrol. Several groups have shown that resveratrol is present in the skin and seeds of grape fruits, as well as the fruits (Melzoch et al. 2001; Romero-Perez et al. 2001; Roldan et al. 2003; Li et al. 2006), and therefore, in wines (Lamuela-Raventos & Waterhouse 1993; McMurtrey et al. 1994; Romero-Perez et al. 1996; Chu et al. 1998; Gu et al. 1999; Melzoch et al. 2001; Wang et al. 2002; Vitrac et al. 2005; Mikes et al. 2008).

An ideal source for resveratrol appears to be grape cane. Resveratrol content from this source has been determined by a number of studies (Karacabey & Mazza 2008; Rayne et al. 2008; Zhang et al. 2011; Vergara et al. 2012; Lambert et al. 2013; Pawlus et al. 2013). These groups have reported resveratrol to be present up to 5.59 mg/g dry weight (DW) from this plant material. Grape cane is generally considered a waste product generated annually when grapevines are pruned. Rayne et al. (2008) estimated the value of resveratrol to be $2,000-3,000/ha of vineyard grape cane. It should not be surprising that research groups have reported various amounts of resveratrol utilizing numerous cultivars of grape cane. Many factors can influence efforts to quantitate resveratrol. Extraction methodology has been explored by Rayne et al. (2008) and Soural et al. (2015).

Which cultivar(s) might be the best choice(s) in a given locale that will yield the greatest resveratrol is clearly another primary concern.

Environmental growing conditions appear to influence resveratrol quantities, even the same cultivars grown in the same vineyard during the same growing season can have very different yields of resveratrol (Vergara et al. 2012). It can be difficult to ascertain the compounding effects of various factors, such as predation, altitude, precipitation, wind currents, and others upon the yield of various plant derived compounds, including resveratrol. Obviously, reporting the median resveratrol from dried canes of a single growing season is not sufficient to optimize the choice of wine grape(s) to grow repeatedly in a specific location.

The objective here is to determine if the cane from certain wine grape cultivar(s) may be better economical choices as source(s) of resveratrol in central Texas. Here we present the relative results of a non-optimized Soxhlet extraction (Whaley et al. 2007) of resveratrol isolated from cane of one growing season. A novel approach to group the ten cultivars was used, and we have tentatively identified a group of wine grapes for further study.

MATERIALS & METHODS

Grape cane waste samples of several varieties were obtained from Texas A&M AgriLife vineyard (Stephenville, TX) during annual pruning activities in February 2010. Extractions began in 2013 and continued into 2014. The dried samples were ground in a Wiley mill to < 1 mm and stored at room temperature before extraction. Extractions were performed by adapting a method employed for isoflavones extraction from Osage orange (Whaley et al. 2007) via soxhlet extraction using hexanes followed by ethyl acetate solvent. This procedure was employed uniformly on all samples and lasted approximately one hr per sample. The solvent from the ethyl acetate extracts was removed under reduced pressure via rotary evaporation and the residue dissolved in methanol. The solutions were transferred to a volumetric flask diluted to volume and stored in a freezer at -10 °C until analysis. Each variety was run in triplicate.

Prior to chromatographic analysis, the samples were allowed to warm to room temperature and filtered through a 0.2 μm Nalgene syringe filter. Analysis was carried out using high performance liquid chromatography (Varian Pro Star) equipped with UV-vis detector and control module. Samples of 20 mL were injected onto a reversed-phase C18 column (Partisil 10 DDS (3), 4.6 by 250 mm; Phenomenex). A gradient solvent system was employed with 50 mM aqueous phosphoric acid (solvent A) and methanol (solvent B) as described previously (Rayne et al. 2008). Thus, the elution profile had the following proportions (v/v) of solvent B: 0 min 10%; 0-30 min 10-25%; 30-50 min 25-45%; 50-55 min 45-100%; 55-60 min 100%; and 60-65 min 100-10%. The solvent flow rate was 1.0 mL/min. Under these conditions, trans-resveratrol eluted at 54 min. Resveratrol's identity was confirmed with an authentic sample obtained from Sigma-Aldrich. Quantitation was performed using an external calibration curve monitoring peak areas at 320 nm.

Beyond median resveratrol concentration in each variety and standard deviation, statistical analysis used the non-parametric Kruskal-Wallis (KW) test (Kruskal & Wallis 1952) of the null hypothesis that all group medians are equal. The more common parametric one-way ANOVA cannot be used because the group sizes (n_i = 3) are not large enough to verify the model assumptions. Following successful rejection of the null hypothesis in the KW test, the post hoc Conover-Iman test with the Bonferroni correction (Conover & Iman 1979) was applied to detect statistically significant pairwise differences between groups. Finally, Akaike Information Criterion (Akaike 1974), Bayesian Information Criterion (Schwarz 1978), and adjusted-r^2 were utilized to identify an optimal balance between model performance and model complexity (the number of distinct groups).

RESULTS

The relative median and standard deviation of the ten varieties was determined and are presented in Table 1. Although an increasing resveratrol concentration in cane waste trend is observed in the different varieties examined, the somewhat large deviations determined from the triplicate analysis of the varieties precludes a prima facie assertion that one variety produces more or less resveratrol than another. The values ranged from 10.3 – 52.3 mg/kgDW for Malavasia Blanca and Lenoir varieties, respectively.

Thus, the non-parametric KW test for equality of group medians was used. The KW test relies on the *H*-statistic, which has the value *H*=18.954 for this data. Unfortunately, the typical method to compute the corresponding *P*-value via approximation by χ^2 distribution is invalid due to sample group sizes (n_i = 3). Moreover, exact KW *P*-values are not available in the literature for this situation due to the computation time required to obtain the complete permutation distribution of *H* under all $\frac{30!}{(3!)^{10}} \cong 4X10^{24}$ permutations. The accepted approach is to create a partial permutation distribution of *H* by randomly sampling a reasonably large number of permutations. Figure 2 shows this distribution using 1,000,000 random permutations along with a vertical line at the observed value *H* = 18.954 for the collected data. The region to the right of the line corresponds to permutations that produced *H* values more extreme than the observed value. It has area 0.004488, which is the *P*-value for this test. Since this is far below any standard significance level (α = 0.05), we strongly reject the null hypothesis that all group medians are equal.

For reference, the *P*-value for the KW test produced by standard software (SciPy) based on approximation methods that are not valid here is 0.025. While higher than the "exact" *P*-value from the more valid approach above, it is still below the standard α = 0.05 and would also reject equality of group medians.

The rejected KW test conclusively demonstrates that the ten grape varieties are not equivalent regarding resveratrol production.

Table 1. Relative median and standard deviation of the ten varieties of grape cane waste.

Variety	Median (mg resveratrol/kgDW cane waste)	Standard Deviation
Malavasia Blanca	10.3	5.84
Canadice	17.0	1.76
Merlot	20.0	13.3
Chardonnay	21.7	9.42
Delicatessen	34.0	7.88
Norton	34.1	12.0
Cabernet Franc	37.6	13.6
Blanca du bois	45.5	26.0
Cabernet Sauvignon	49.6	9.66
Lenoir	52.3	35.1

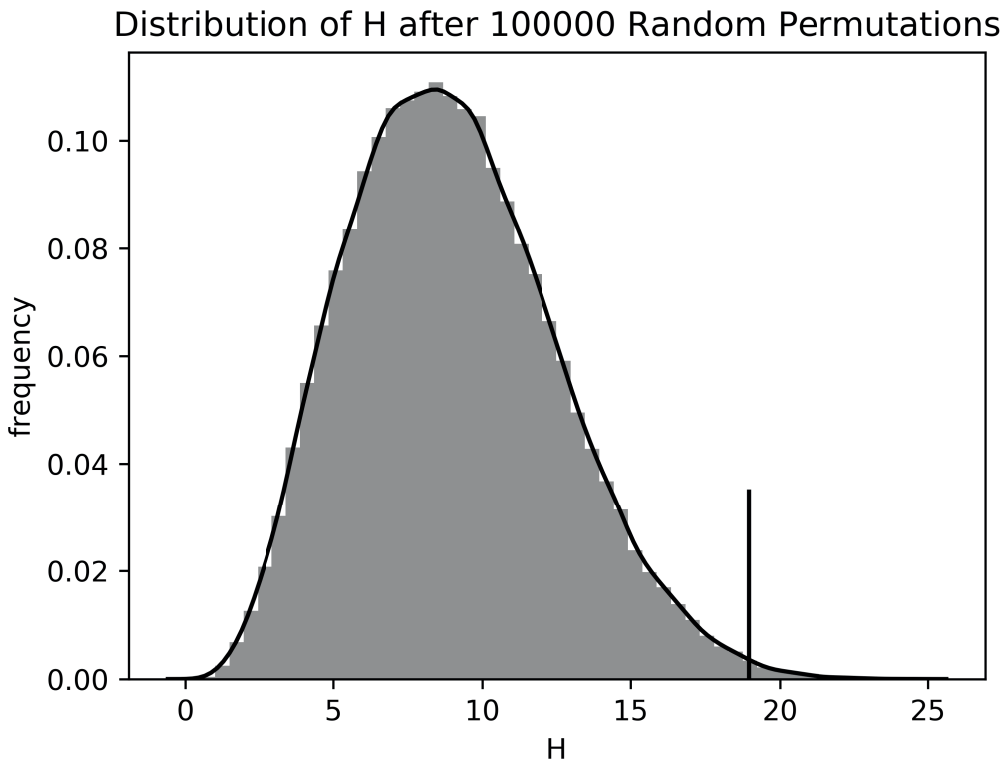

Figure 2. Distribution of H after 100000 Random Permutations. The vertical line represents the *H* from the data measured.

Table 2. Post Hoc Pairwise Difference for Malavasia Blanca (Mal), Canadice (Can), Merlot (Mer), Chardonnay (Char), Delicatessen (Del), Norton (Nor), Cabernet Franc (CF), Blanca du bois (Bdb), Cabernet Sauvignon (CS), Lenoir (Len).

	Mal	Can	Mer	Char	Del	Nor	CF	Bdb	CS	Len
Mal		1.00	1.00	1.00	1.00	0.27	1.00	0.42	0.03(*)	0.01(*)
Can	1.00		1.00	1.00	1.00	1.00	1.00	1.00	0.23	0.08
Mer	1.00	1.00		1.00	1.00	1.00	1.00	1.00	0.36	0.13
Char	1.00	1.00	1.00		1.00	1.00	1.00	1.00	0.36	0.13
Del	1.00	1.00	1.00	1.00		1.00	1.00	1.00	1.00	1.00
Nor	0.27	1.00	1.00	1.00	1.00		1.00	1.00	1.00	1.00
CF	1.00	1.00	1.00	1.00	1.00	1.00		1.00	1.00	1.00
Bdb	0.42	1.00	1.00	1.00	1.00	1.00	1.00		1.00	1.00
CS	0.03(*)	0.23	0.36	0.36	1.00	1.00	1.00	1.00		1.00
Len	0.01(*)	0.08	0.13	0.13	1.00	1.00	1.00	1.00	1.00	

Following KW rejection, it is standard to apply a post hoc test to detect pairwise differences between groups. We applied the Conover-Iman test with the Bonferroni correction against multiple comparisons. The only significant ($\alpha = 0.05$) pairwise differences were Malavasia-Cabernet Sauvignon and Malavasia-Lenoir (Table 2).

Interestingly, an eleventh sample (*Vitis arizonica* grape cane) yielded 202 mg/kgDW resveratrol, however insufficient material was available for triplicate extractions. Rayne et al. (2008) and Karacabey & Mazza (2008) used only Pinot Noir, and Vergara et al. (2012) focused mostly upon Pinot Noir and Gewurztraminer. Of the ten varieties utilized in this study, none were Pinot Noir. It is possible that Pinot Noir cane is a candidate for resveratrol extraction.

DISCUSSION

Soural et al. (2015) showed that various conditions, such as solvent, preparation of tissue, temperature of extraction, extraction procedure, could influence yields of resveratrol from grape cane. Of the recommended factors, our procedure included only a Soxhlet extraction of eight cycles. As a consequence, our results with grape cane as source material are approximately 15- to 60-fold less than those reported by Rayne et al. (2008), Karacabey & Mazza (2008),

and Vergara et al. (2012). Rayne et al. (2008) reported 3,450 mg/kgDW resveratrol from Pinot Noir, where Vergara et al. (2012) obtained 3,676 and 4,628 mg/kgDW from Pinot Noir and Gewurztraminer, respectively. Karacabey & Mazza (2008) achieved 4,250 mg/kgDW resveratrol from Pinot Noir. More pointedly our Cabernet Sauvignon and Merlot values are 17.6- and 59-fold lower than those reported by Lambert et al. (2013), who used a water-acetone solvent for extraction. As well, Pawlus et al. (2013) reported a 32.7-fold greater value for resveratrol from Cabernet Sauvignon.

The solvents of 80:20 and 70:30 ethanol:water of Rayne et al. (2008), or methanol (Soural et al. 2015) appear to be the preferred solvents for extraction of resveratrol and other stilbenes from grape cane. The ethyl acetate solvent employed in this study is of intermediate efficacy for resveratrol extraction below acetone, ethanol and methanol, but superior to ether, chloroform and water.

Storage of the tissue after pruning can also influence the yields of stilbenes. Gorena et al. (2014) found that some stilbenes, including resveratrol, will accumulate up to two-months post harvest. Also, this group found that cultivar Gewurztraminer can yield resveratrol at a concentration of 5,959 mg/kgDW after three months of storage (Gorena et al. 2014). The data of Houille et al. (2015) suggest that resveratrol concentrations do not change dramatically up to eight months. Additionally, a considerable amount of resveratrol can be extracted from year old grape canes (Zhang et al. 2011). However, no reports of three years of storage at room temperature on resveratrol yields have appeared. It is very possible that resveratrol has degraded significantly in that time.

At least one report exists documenting the effect of storage temperature of grape cane upon yields of resveratrol. Houille et al. (2015) showed that the optimum storage temperature is approximately 20 ^{o}C. The grape cane in this study was stored at room temperature (22 ^{o}C) until extraction three years later.

Year-to-year environmental growing conditions also appear to influence resveratrol quantities. Pinot Noir grown at the same vineyard on two successive years yielded 723 and 2,551 mg/kgDW (Vergara et al. 2012). The authors also found very different yields of resveratrol for samples within the same growing year. Different vineyards could yield resveratrol anywhere from 2,551–5,590 mg/kgDW, and for Gewurtraminer the yield was 3,275–6,533 mg/kgDW (Vergara et al. 2012). These environmental factors include, but are not limited to altitude, intensity of the sun's radiation, and predation by herbivores. Even within the same vineyard in the same growing season, the resveratrol yield can be dramatically different (Vergara et al. 2012).

We explored a conjecture that the 10 varieties can be grouped in larger "families" with similar resveratrol production characteristics. There are 115,974 unique ways to group 10 objects into two or more groups, allowing unevenly-sized groups. Our algorithm loops over all such groupings, fits the best ordinary linear model (via python statsmodels ols), and applied the model evaluation metrics Akaike Information Criterion (AIC), Bayesian Information Criterion (BIC), and adjusted-r^2. Recall, better models receive *smaller* AIC and BIC values, but *larger* adjusted-r^2 values.

Surprisingly, all three of these metrics basically agree, grouping as follows: 1. Malavasia, Canadice, Merlot, Chardonnay; 2. Delicatessen, Cabernet Franc; 3. Norton, Blanca Du Bois, Cabernet Sauvignon; and 4. Lenoir.

Both adjusted-r^2 and AIC produce this exact grouping; BIC produces this grouping but combines groups two & three. Table 3 shows the top 10 groupings. It should be reiterated that, because there are only three observations of each variety, there is no reliable way to assess regularity of residuals and variances across groups.

Since this study is preliminary in nature and the yield of resveratrol is not absolute, this study did not determine whether central Texas is an ideal location for growth of grapevines for extraction of maximum

Table 3. The top 10 groupings based on AIC (Akaike Information Criterion), BIC (Bayesian Information Criterion) and adjusted-r^2 analysis. "Grouping" column assigns varietals to groups in the following order: Malavasia Blanca, Canadice, Merlot, Chardonnay, Delicatessen, Norton, Cabernet Franc, Blanca du bois, Cabernet Sauvignon, Lenoir.

Grouping	# groups	adjusted r^2	AIC	BIC
[0000121223]	4	0.55984	250.72	256.33
[0000121234]	5	0.55498	251.88	258.88
[0111232334]	5	0.55474	251.89	258.90
[0011232334]	5	0.55230	252.06	259.06
[0000111223]	4	0.55088	251.33	256.93
[0111232345]	6	0.54947	253.02	261.43
[0000111123]	4	0.54906	251.45	257.05
[0010121223]	4	0.54733	251.56	257.17
[0011232345]	6	0.54692	253.19	261.60
[0000121334]	5	0.54692	252.41	259.42

amounts of resveratrol. Environmental factors are too varied to draw that conclusion from data of one growing season. Rather the purpose was to tentatively identify grape cultivar(s) that could be worthy candidates to reliably yield a sizeable, economical amount of resveratrol each year in central Texas. To this end, we developed a new approach of statistical analysis that will concentrate efforts towards selection of these four grape varieties.

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