

## Stilbene Levels in Grape Cane of Different Cultivars in Southern Chile: Determination by HPLC-DAD-MS/MS Method

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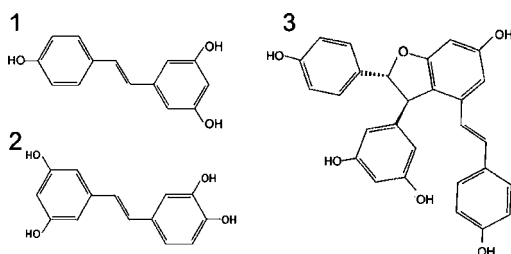
**ABSTRACT:** Health benefits of *trans*-resveratrol and other stilbenes in grapes, must, and wine have been pointed out by numerous authors. Less attention has been paid to the presence of stilbene derivatives in viticultural residues, such as grape canes. The present work reports the first results of a systematic study of stilbene levels in different grape varieties and cultivation areas in Chile, to evaluate their potential as an alternative source of bioactive stilbenes. In all cane samples, the predominant stilbene is *trans*-resveratrol, followed by *ε*-viniferin and piceatannol. In canes of Pinot noir up to  $5590 \pm 172 \text{ mg kg}^{-1}$  of *trans*-resveratrol and up to  $6915 \pm 175 \text{ mg kg}^{-1}$  of total stilbenes were detected. The observed concentrations of stilbenes in canes of Pinot noir from southern Chile until now are higher than those reported previously for this red variety. However, the highest concentration of total stilbenes observed in the analyzed samples was in the canes of white variety Gewürztraminer with  $7857 \pm 498 \text{ mg kg}^{-1}$ . Preliminary results indicate that these levels can evolve if canes are left for some months on the vineyard after pruning, observing an increase during the first 2 months and a decrease after this period.

**KEYWORDS:** stilbenes, grape cane, *Vitis vinifera*, Chile

### INTRODUCTION

The increasing evidence of health benefits of *trans*-resveratrol triggered in the past two decades the interest in studying levels of this and other stilbenes in grapes, must, and wine.<sup>1</sup> However, less attention has been paid to the presence of stilbenes in viticultural residues, such as grape canes, that is, the lignocellulosic residue generated during the annual pruning of grapevines (*Vitis vinifera*). Commonly, grape cane is incorporated into the soil of the vineyard, transformed to charcoal, or burned. Stilbenes are secondary metabolites that act as phytoalexins<sup>2</sup> in grapevine. They are known to occur in wood, leaves, stems (rachis and pedicels), and berries.<sup>3,4</sup>

Members of the stilbene family possess a carbon skeleton based on the *trans*-resveratrol (3,5,4'-trihydroxystilbene) structure, including, for example, piceids, pterostilbenes, and viniferins, which are, respectively, glucosides, dimethylated derivatives, and oligomers of resveratrol (Figure 1).



**Figure 1.** Chemical structures of principal stilbenes found in grape cane samples from southern Chile: (1) *trans*-resveratrol; (2) *trans*-piceatannol; (3) *ε*-viniferin.

Resveratrol has attracted considerable research interest due to its postulated cardioprotective effects when it is present in

red wine.<sup>5</sup> Since then, several articles and reviews<sup>6,7</sup> have shown that resveratrol can prevent or slow the progression of a wide variety of degenerative diseases, including cancer<sup>8–10</sup> and cardiovascular disease.<sup>11</sup>

Whereas in several publications the levels of resveratrol and other stilbenes in wine,<sup>12–14</sup> grapes,<sup>1,15,16</sup> and grape skin<sup>14</sup> have been reported, only a few papers have dealt with the vegetative part of the plant, that is, grape cane, leaves, and stems, as an additional source for these high-value bioactive stilbenes. Püssa et al.<sup>17</sup> made a survey of grapevine stem polyphenols, including resveratrol, *ε*-viniferin, and the main trimeric stilbenes. They reported resveratrol concentrations between 1100 and 3300 mg kg<sup>-1</sup> dry weight. The sum of the main stilbenoids in stems of three *V. vinifera* varieties cultivated in Estonia was in the range of 1800–5100 mg kg<sup>-1</sup>. In the case of grape cane, Rayne et al.<sup>18</sup> reported levels of 3450 and 1300 mg kg<sup>-1</sup> dry weight of *trans*-resveratrol and *ε*-viniferin, respectively, in grape cane waste of Pinot noir from British Columbia in Canada. These data clearly revealed that stilbene concentrations in these so far not exploited residues can be significantly higher compared to wine, grapes, leaves, or cluster stems.

The stilbene concentrations range between 2 and 5 g kg<sup>-1</sup> dry weight in grapevine cane, and according to Rayne et al.<sup>18</sup> the commercial value of resveratrol is between U.S. \$2000 and U.S. \$3000 per kilogram. Stilbene yields from cane waste could represent an agricultural byproduct valued between U.S. \$2000 and U.S. \$3000 per hectare.<sup>18</sup> However, a recent Web search of market prices for bulk grape resveratrol extracts from grapes

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showed a range between U.S. \$300 and U.S. \$970 for 1 kg of resveratrol, depending of its origin and degree of purity.<sup>19,20</sup>

To the best of our knowledge, at international and national levels, no systematic study of the levels of stilbenoids in grapevine canes of the grape varieties commonly cultivated in Chile, except Pinot noir, is available until now.

The present work reports the first result of a study of stilbene levels in different grapevine varieties and cultivation areas in Chile, to evaluate their potential as an alternative source for the isolation of bioactive stilbenes.

## MATERIALS AND METHODS

**Chemicals.** All solutions were prepared in 18 M $\Omega$  deionized water from a Millipore Milli-Q water purification system. HPLC grade acetonitrile and water, ethanol, and formic acid were purchased from Merck (Darmstadt, Germany). *trans*-Resveratrol standard (99% purity) was obtained Phytolab (Vestenbergsgreuth, Germany).

**Samples.** Cane samples were collected in the Bio-Bio region in southern Chile during the pruning seasons (July–August 2009 and 2010) from two vineyards located in the Itata valley, Ránquil (36° 34' 47" S/72° 41' 32" W) and Bulnes (36° 46' 42" S/72° 13' 24" W), and from three vineyards in the Bío-Bío valley, Patagual (37° 01' 42" S/72° 58' 57" W), Negrete (37° 38' 48" S/72° 12' 41" W), and Mulchén (37° 38' 48" S/72° 12' 41" W). All collected grape cane samples presented no visual evidence of damage due to fungal attack.

**Extraction Method.** The collected cane samples were stored in a fresh, dry, and dark place during a period of 2–4 months until they were chopped to smaller pieces with a pruning scissor and then ground to a particle size below 20 mesh with a hammer mill (Condux-Werk LS 10M; Wolfgang/Hanau, Germany). The ground samples were stored at room temperature in hermetic plastic bags protected from light exposure.

As extraction solvent an ethanol/water mixture (80:20 v/v) was used,<sup>21</sup> but with major modifications in the procedure. The requirement of a previous defatting process was evaluated, too. The method after optimization was the following: 2.0 g of sample was extracted four times with 16.0 mL of an ethanol/water (80:20 v/v) mixture using a Cole-Parmer 4710 series ultrasonic bar (Chicago, IL) with a frequency of 50 Hz at room temperature during 5 min for each extraction. After solvent evaporation with a nitrogen flow and protection of the extract from light exposure, the dried extracts were reconstituted in a mixture of 85% mobile phase A and 15% mobile phase B and then passed through a GV Durapore filter (0.22 mm pore size, 13 mm diameter, Millipore, Bedford, MA) before chromatographic analysis. The water content of each sample was determined in parallel to stilbene analysis, drying 2.0 g of the ground grape cane sample at 100 °C until a constant weight was reached. The calculated percentage of moisture was used to correct the stilbene concentration of each sample to a dry weight basis, to facilitate the comparison of samples with different degrees of moisture.

**Chromatographic Methods.** The HPLC-DAD-ESI-MS/MS analyses of stilbenes were carried out with a HPLC system (Shimadzu Co., Japan) equipped with a quaternary LC-10ADVP pump, an FCV-10ALVP elution unit, a DGU-14A degasser unit, and a CTO-10AVP oven. A UV-vis diode array spectrophotometer Shimadzu model SPD-M10AVP in tandem with a mass spectrometer 3200 QTRAP (Applied Biosystems, USA) was used, with the control system and data collection CLASS-VP software for DAD and Analyst software (version 1.5.2) for MS/MS analysis.

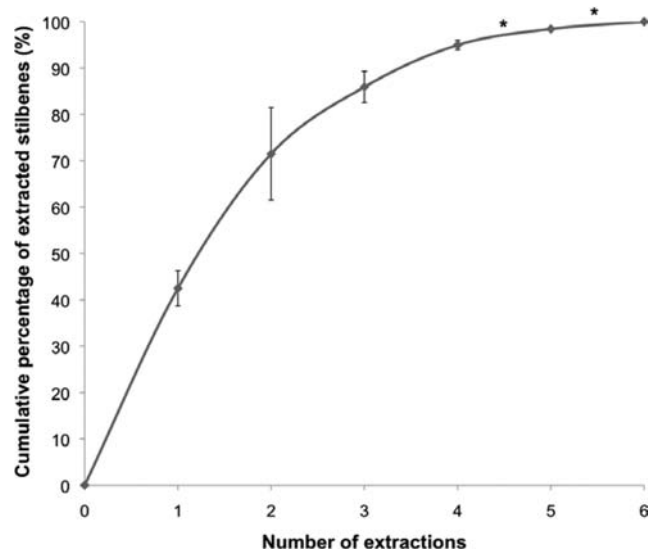
HPLC analyses of stilbenes were carried out on a C18 Kromasil 5  $\mu$ m, 250  $\times$  4.6 mm, column (Akso Nobel, Bohus, Sweden) with a C18, Nova-Pak Waters, 22  $\times$  3.9 mm, 4  $\mu$ m precolumn (Milford, MA) at 30 °C. The injection volume was 25  $\mu$ L. The analysis of stilbenes was carried out using a mobile phase gradient constituted by formic acid 0.1% v/v (solvent A) and acetonitrile (solvent B),<sup>17</sup> but adapting the mobile phase gradient. The flow rate was 0.5 mL min<sup>-1</sup>, and the gradient program was from 15 to 20% of solvent B in 5 min, from 20 to 44.5% in 45 min, then up to 100% in 1 min, and kept at 100% of B

during 9 min, followed by 5 min of stabilization at 15%. The spectrophotometric detection was set at 306 nm. Quantification was performed using an external calibration curve with *trans*-resveratrol as external standard and expressing results as *trans*-resveratrol equivalents.

Identity assignment of stilbenes was made by ESI-MS/MS employing the following parameters: negative ionization mode; drying temperature, 450 °C; ion spray voltage, -4000 V; nebulizer gas, 40 psi; auxiliary gas, 50 psi; scan range, *m/z* 100–1200. The chromatographic conditions were the same as mentioned above.

## RESULTS AND DISCUSSION

**Extraction and Analytical Method Parameters.** Before the stilbene profile in grape canes could be determined, the extraction method had to be optimized by evaluating the solid-liquid ratio, ethanol percentage, number of extraction steps, and the need for defatting steps with hexane. The optimization was made with a face-centered central composite design. The optimum condition reached is detailed under Materials and Methods. The extraction method was tested in consecutive extractions leading to 95% of total stilbenes recovered after four consecutive extractions with an ethanol/water (80:20 v/v) mixture using an ultrasonic bar (Figure 2). The repeatability



**Figure 2.** Total stilbene extraction curve from grape cane expressed as accumulated percentage of extracted stilbenes. Asterisks indicate no significant ( $\alpha = 0.05$ ) increase in the quantity of *trans*-resveratrol in subsequent extraction steps. Error bars correspond to the standard deviation of the replicates.

was evaluated with real samples subjected to the overall method including sample preparation, preconcentration, and chromatographic analysis during a period of 10 days resulting in an intermediate precision of RSD 9%, which is appropriate for an extraction method with numerous steps and involving light-sensitive compounds. The limits of detection and quantification for *trans*-resveratrol were 62 and 207 mg kg<sup>-1</sup>, respectively. For stilbene quantification, each sample was extracted in triplicate, and HPLC-DAD analyses were made in duplicate. No relevant interferences were observed when grape cane samples were extracted directly, without previous defatting with hexane.

**Stilbene Composition in Grape Cane.** The major stilbene compounds found in the grape cane extract were *trans*-resveratrol, followed by *e*-viniferin and *trans*-piceatannol. The only conjugate found was *trans*-piceid in very small

proportions along with trace levels of dimers (peaks 4 and 8), a trimer (peak 7) of resveratrol, and the dimeric derivative ampelopsin A (peak 2). The *cis*-isomer of resveratrol was not detected in grape cane. Table 1 shows the stilbene profile in a

**Table 1. HPLC-DAD-MS/MS of Stilbenes in Pinot noir Grape Cane Sample**

stilbene	peak	$t_R$ (min)	$\lambda_{max}$ (nm)	pseudomolecular ion $[M - H]^-$	product ions
<i>trans</i> -piceid	1	21.2	304 (315)	389	227
ampelopsin A	2	25.3	traces	469	452, 408, 375, 345, 335
<i>trans</i> -piceatannol	3	26.5	323 (303)	243	
dimer	4	29.5	328	453	359, 265
<i>trans</i> -resveratrol	5	34.6	304 (316)	227	
$\epsilon$ -viniferin	6	45.7	322 (308)	453	347
trimer	7	47.2	traces	679	
dimer	8	48.7	316 (307)	453	436, 304

grape cane extract, which was the same for all of the varieties under study. Peaks 4 and 8 show a molecular ion  $[M - H]^-$  of 453 amu, which suggested a dimeric structure, different from  $\epsilon$ -viniferin, whereby peak 8 is most likely  $\delta$ -viniferin. Peak 7 shows a molecular ion  $[M - H]^-$  of 679 amu that corresponds

to a trimer, probably one of the isomers of miyabenol C, recently described by Mattivi et al. in *V. vinifera* leaves.<sup>22</sup> In reversed phase HPLC it elutes between  $\epsilon$ -viniferin (peak 6) and the dimer (peak 8), and mass spectra are similar, but NMR analyses are required to confirm the structure.

**Stilbene Concentration in Different Varieties of Grape Cane.** For the stilbene quantification only the three major stilbenes (i.e., *trans*-resveratrol,  $\epsilon$ -viniferin, and *trans*-piceatannol) were considered, mainly because the other stilbenoids detected were below the quantification limit of the implemented methodology. Total stilbene concentration was considered as the sum of the concentration of these three major stilbenes, quantified as *trans*-resveratrol equivalents.

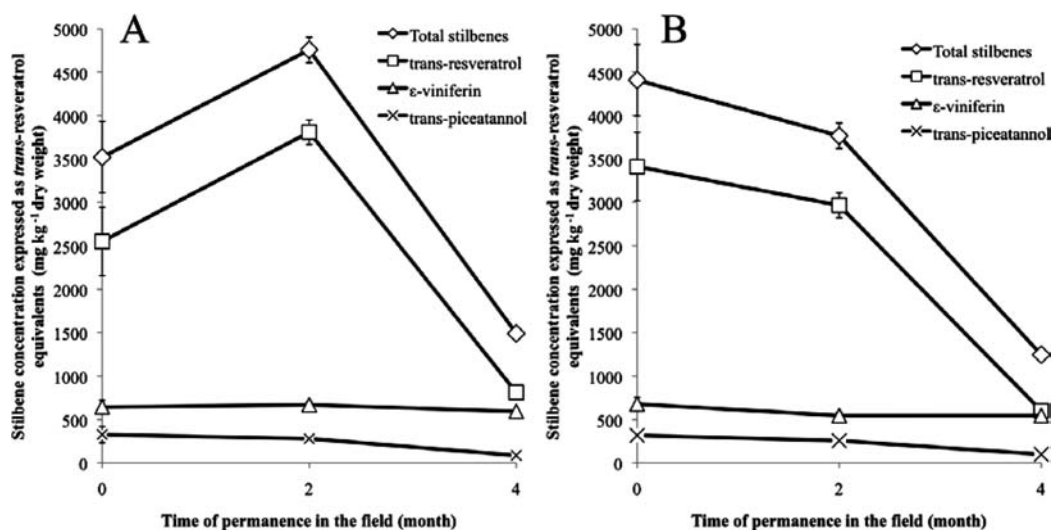
In Table 2 the concentrations of the major stilbenoids are presented. The highest mean concentration of *trans*-resveratrol in the collected samples was determined for Gewürztraminer canes ( $4628 \pm 568 \text{ mg kg}^{-1}$ ), followed by Pinot noir ( $3676 \pm 353 \text{ mg kg}^{-1}$ ). These concentrations are higher than the published concentrations for *trans*-resveratrol and  $\epsilon$ -viniferin; the average level of *trans*-resveratrol was around  $3000 \text{ mg kg}^{-1}$  and that of  $\epsilon$ -viniferin,  $1300 \text{ mg kg}^{-1}$ , in Pinot noir canes.<sup>18,21,23</sup> The variation of the  $\epsilon$ -viniferin level was not significant ( $\alpha = 0.05$ ) in the overall variation of total stilbenes, for which the main contribution can be attributed to the variations in *trans*-resveratrol concentration.

The highest concentration of total stilbenes, expressed as *trans*-resveratrol equivalents, was found in a Gewürztraminer

**Table 2. Stilbene Concentrations in Grape Canes from Vineyards in the Bío-Bío Region of Southern Chile**

sample	stilbene concentrations expressed as <i>trans</i> -resveratrol equivalents <sup>a</sup> (mg kg <sup>-1</sup> of dry weight)			
	<i>trans</i> -piceatannol	<i>trans</i> -resveratrol	$\epsilon$ -viniferin	total
<b>Pinot noir</b>				
Ránquil 2009	125 ± 4a	723 ± 128a	643 ± 29a	1492 ± 143a
Negrete 2009 plot 1	259 ± 4b	4040 ± 140b	714 ± 34a	5013 ± 144b
Ránquil 2010	327 ± 96b	2551 ± 392c	644 ± 77a	3521 ± 411c
Patagual 2010	402 ± 56b	4335 ± 926b	745 ± 158a	5482 ± 942b
Negrete 2010 plot 1	72 ± 11a	2806 ± 674c	433 ± 103b	3312 ± 682c
Negrete 2010 plot 2	154 ± 50a	4072 ± 313b	729 ± 25a	4955 ± 318b
Negrete 2010 plot 3	404 ± 264b	3950 ± 456b	812 ± 83a	5166 ± 534b
Negrete 2010 plot 4	203 ± 7b	4369 ± 131b	704 ± 26a	5275 ± 134b
Negrete 2010 plot 5	458 ± 7c	5590 ± 172d	868 ± 28a	6915 ± 175d
Bulnes 2010	324 ± 13b	4209 ± 190b	660 ± 27a	5193 ± 192b
Mulchén 2010	398 ± 55b	3789 ± 360b	744 ± 75a	4931 ± 372b
mean	284 ± 52	3676 ± 353*	700 ± 60*	4660 ± 368*
<b>Gewürztraminer</b>				
Patagual 2010	530 ± 45a	4075 ± 312a	824 ± 72a	5429 ± 323a
Negrete 2010	615 ± 59a	6533 ± 488b	709 ± 80a	7857 ± 498b
Quitralman 2010	226 ± 12b	3275 ± 905a	700 ± 162a	4201 ± 919a
mean	457 ± 38	4628 ± 568*	744 ± 105*	5829 ± 580*
<b>other varieties</b>				
Cabernet Sauvignon Ránquil 2009	106 ± 11	484 ± 14	346 ± 28	936 ± 33
Cabernet Sauvignon Ránquil 2010	318 ± 19	3413 ± 251	679 ± 56	4409 ± 258
Tintorera Ránquil 2009	83 ± 1	383 ± 12	75 ± 10	540 ± 16
Tintorera Ránquil 2010	275 ± 16	4279 ± 104	642 ± 1	5195 ± 105
Cinsault Ránquil 2009	90 ± 3	446 ± 7	266 ± 4	803 ± 9
Moscatel de Alejandria Ránquil 2009	97 ± 11	1038 ± 79	316 ± 41	1451 ± 90
Zinfandel Bulnes 2010	91 ± 27	1085 ± 18	462 ± 2	1639 ± 33
Carménère Bulnes 2010	186 ± 4	2493 ± 155	414 ± 20	3093 ± 157
Malbec Bulnes 2010	235 ± 3	3589 ± 485	603 ± 91	4428 ± 553

<sup>a</sup>Lower case letters indicate significant difference ( $\alpha = 0.05$ ) for the concentrations of stilbenes found for each variety (Scheffé test). The asterisk indicates significant difference ( $\alpha = 0.05$ ) between Pinot noir and Gewürztraminer varieties for the mean concentrations of stilbenes.



**Figure 3.** Stilbene concentration in grape canes from Pinot noir (A) and Cabernet Sauvignon (B) left in the open field in the vineyard after 4 months. Error bars correspond to the standard deviation from three extractions.

sample from Negrete 2010,  $7857 \pm 498$  mg kg<sup>-1</sup>, and the second highest was a Pinot noir,  $6915 \pm 175$  mg kg<sup>-1</sup>, from the same vineyard (Negrete 2010 plot 5). The two above-mentioned samples with the highest concentrations of stilbenes, namely Gewürztraminer Negrete 2010 and Pinot Noir Negrete 2010 plot 5, were also the samples that were collected last, so they were analyzed a few days after pruning; meanwhile, the samples that were collected first had a longer period of storage in the laboratory. This observation requires further evaluation.

Five Pinot noir samples were collected from different plots of the vineyard in Negrete (Negrete 2010, plots 1–5). It can be observed (Table 2) that in the same vineyard significantly different concentrations of stilbenoids can be detected ( $\alpha = 0.05$ ). Whereas between three samples of different plots there was no significant difference ( $4955 \pm 318$  to  $5275 \pm 134$  mg kg<sup>-1</sup>), one had a higher ( $6915 \pm 175$  mg kg<sup>-1</sup>) and the other a lower ( $3312 \pm 682$  mg kg<sup>-1</sup>) stilbene concentration. This observation must lead to further investigations about the influence of the variations in the terroir and other factors that may have an effect on the stilbene concentration in grape canes.

The lowest concentrations of total stilbenes were found in Pinot noir, Cabernet Sauvignon, Tintorera, Cinsault, and Moscatel de Alejandria collected in Ránquil in 2009, with concentrations being below 1500 mg kg<sup>-1</sup>. The stilbene levels found in 2009 were much lower, compared with the samples taken at the same locations in 2010. This behavior was not observed for the Pinot noir sample collected from Negrete 2009 (plot 1), which was higher than the sample from the same plot in that vineyard in 2010. The higher content of stilbenes in 2010 could be attributed to an increased synthesis of these compounds due to a response to injury or infection of the plant.<sup>18,21,24–26</sup> Another factor that must be considered is the influence of the time elapsed between sample collection and the actual analysis. To determine the influence of the time between pruning/collection and analysis, a preliminary assay was performed in 2010 for Pinot noir and Cabernet Sauvignon grape canes. In this experiment, the grape canes were left in the vineyard after pruning, exposed to the environment for a period of 4 months (Figure 3). In the case of Pinot noir in the first 2 months, the resveratrol concentration increased by 35% from the initial concentration of total stilbenes and later dropped to

almost half of the initial level until the end of the fourth month. In Cabernet Sauvignon the levels of total stilbenes decreased to a concentration similar to the last found in Pinot noir. The ε-viniferin concentration in both varieties maintained a relatively stable level after pruning. *trans*-Piceatannol levels showed the same behavior in both varieties, without any significant variation.

Very recently *trans*-resveratrol levels in grape canes, mainly of local varieties from Turkey<sup>27</sup> and China,<sup>28</sup> were published. The mean *trans*-resveratrol concentration found in several grape canes cultivated in Turkey was 0.022 mg kg<sup>-1</sup>.<sup>27</sup> Much higher concentrations were found in 1-year-old grape canes from *V. vinifera* cultivated in China, with a *trans*-resveratrol content that ranged from 664 to 1751 mg kg<sup>-1</sup>,<sup>28</sup> these levels are closer to the concentration of *trans*-resveratrol reported by Püssa et al.,<sup>17</sup> Rayne et al.,<sup>18</sup> and Karacabey and Mazza<sup>21</sup> of 2100, 3450, and 4250 mg kg<sup>-1</sup>, respectively, and lower than the *trans*-resveratrol concentrations range found in this work.

The proposed ultrasound-assisted extraction and stilbene determination method by HPLC-DAD-ESI-MS/MS is appropriate to determine stilbene levels in grape cane.

The concentration ranges of *trans*-resveratrol and total stilbenes found in most of the grape cane samples from vineyards in the Bio-Bio region in Chile were higher than those previously reported in the literature. This can be attributed in part not only to a better extraction recovery employing an ultrasonic bar but also to environmental factors that can have an effect on increased induction of biosynthesis mediated by elicitors.

*V. vinifera* canes from southern Chile show an interesting potential as a source of stilbenes for industrial use. However, a more detailed study that considers incidence of diseases and collection time is required to estimate stilbene variation in the grape canes after pruning and the impact of these factors on the extraction yields of these bioactive compounds.

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