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Determination of Stilbenes (δ-viniferin, *trans*-astringin, *trans*-piceid, *cis*- and *trans*-resveratrol, ε-viniferin) in Brazilian Wines

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Phenolics from grapes and wines can play a role against oxidation and development of atherosclerosis. Stilbenes have been shown to protect lipoproteins from oxidative damage and to have cancer chemopreventive activity. We describe a method for the direct determination of stilbenes in several red wines using high-performance liquid chromatography with UV detection. In a survey of 12 commercial wines from the south of Brazil (Rio Grande del Sul), levels of δ-viniferin are reported for the first time in different varieties of red wines. Brazilian red wine contains *trans*-astringin, *trans*-piceid, *trans*-resveratrol, *cis*-resveratrol (in high quantity: 5 times more than the trans form), ε-viniferin, and a compound isolated for the first time in wine, *trans*-δ-viniferin. Isolation and identification of δ-viniferin was achieved by NMR after extraction and fractionation of red wine phenolics. δ-Viniferin contributes, as well as *cis*-resveratrol and *trans*-piceid, to a significant proportion of stilbenes in wine dietary intake, particularly with Merlot varieties containing an average level of 10 mg/L for δ-viniferin, 15 mg/L for *cis*-resveratrol, and 13 mg/L for *trans*-piceid. The total stilbene intake from wine origin was estimated for the Brazilian population as 5.3 mg/day per person (on the basis of a regular wine consumption of 160 mL/day). δ-Viniferin can contribute to around 20% of total stilbenes in wine (average of 6.4 mg/L in red Brazilian wines). It would be important in the future to investigate the origins of the differences in wine stilbene levels in relation to the vine varieties, and the bioavailability of the newly extracted stilbene δ-viniferin in plasma after consumption of different types of wines.

KEYWORDS: Brazilian red wine; varieties; stilbenes; ε-viniferin; δ-viniferin; *cis*- and *trans*-resveratrol; *trans*-piceid; NMR; HPLC−UV

INTRODUCTION

Stilbenes occur naturally in various families of plants, but grapes and related products are considered the most important dietary sources of these substances (*1*). Epidemiological studies have shown that moderate wine consumption is related to a decrease in cardiovascular diseases, cancer (*2*, *3*), and dementia (*4*). It has been hypothesized that the phenolic substances of red wine may be responsible for these potential beneficial effects by potent antioxidant properties (*5*−*7*).

Among stilbene monomers, *trans*-resveratrol has been the most widely studied grapevine phytoalexin for its role on human heath, although a low concentration of this compound (1−3 mg/L) was found in wine (*8*, *9*). However, the level of its 3-*O*-β-glucoside, *trans*-piceid, reached maximal concentrations of about 30 mg/L in French wines (*9*, *10*). Its deglycosylation by small intestine epithelial β-glucosidases may occur, as shown for flavonoids (*11*). *trans*-Resveratrol has been suggested as one of the components in red wine that may be beneficial to human cardiovascular health. Indeed, it has been reported to inhibit platelet aggregation (*12*) and LDL oxidation (*13*), to produce endothelial nitric oxide-dependent vasorelaxation in vivo (*14*) and to inhibit the proliferation of vascular smooth muscle cells (*15*). With regard to cancer, *trans*-resveratrol inhibits the

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proliferation of tumor cells and has a cancer-chemopreventive potential, inhibiting cellular events associated with the three major stages of carcinogenesis (*16*, *17*). Moreover, it could play the role of prodrug since it is hydroxylated in human liver into *trans*-piceatannol, a compound with known anticancer properties through the inhibition of tyrosine kinase and the induction of apoptosis (*18*). The latter may be also produced by intestinal hydrolysis of *trans*-astringin which is sometimes found at higher concentrations than *trans*-piceid in wines (*9*, *10*). *trans*-Astringin itself was reported to have potential cancer-chemopreventive activity (*17*).

cis-Resveratrol and *cis*-piceid are typically found at lower concentrations and are often less biologically active than trans forms (*9*, *10*, *12*). Some resveratrol dimers have been characterized in Riesling wine at very low levels (<0.05 mg/L) such as ϵ-viniferin diglucosides and pallidol mono- and di-glucosides (*19*), and in red wines at low levels (from 0.5 mg to 3 mg/L) such as ϵ-viniferin, parthenocissin A, and pallidol (*10*, *20*, *21*). Moreover, a resveratrol *trans*-dehydrodimer (δ-viniferin) and its 11- and 11′-β-*O*-glucosides have been demonstrated to be constituents of *Vitis vinifera* cell suspension cultures (*17*). Recently, *trans*-δ-viniferin was shown to be one of the major stilbenes synthesized by *Plasmopora viticola*-infected grapevine leaves (*22*) and to exhibit inhibitory activity against cyclooxygenase-1 and -2 with IC_{50} values in the range of 5 μM (*17*).

We report the NMR characterization of *trans*-δ-viniferin and its concentration in commercial red wines from Brazil. The contents of *trans*- and *cis*-resveratrol, *trans*-piceid, *trans*-astringin, and ϵ-viniferin were also determined using a high-performance liquid chromatography (HPLC) method coupled with UV detection.

MATERIAL AND METHODS

Reagents. Methanol and acetonitrile (HPLC grade) were purchased from Carlo Erba (Val de Reuil, France) and Merck (Nogent sur Marne, France).

Standards. *trans*-Resveratrol was purchased from Sigma (St. Quentin Fallavier, France). *trans*- and *cis*-Piceid and *trans*-astringin were extracted from grape cell suspension cultures (*23*−*25*). ϵ-Viniferin was extracted from Merlot stalks (*26*). *cis*-Resveratrol was obtained after hydrolysis of *cis*-piceid with a β-glucosidase (*24*). β-Glucosidase digestion of the *cis*-piceid was performed. The methanol was removed under nitrogen and the residue dissolved in 200 μL of 25 mM citric acid/phosphate buffer (pH 5.2) containing 0.5 mg of β-glucosidase from almonds per mL (Sigma Chemical, St. Louis, MO). After incubation at 37 °C for 1 h, the free resveratrol was extracted three times from the aqueous phase with an equal volume of ethyl acetate. The ethyl acetate extracts were pooled and concentrated to dryness under nitrogen, dissolved in methanol, and then analyzed by HPLC. The identity of resveratrol was confirmed by cochromatography with a *cis*-resveratrol standard and by comparison of UV absorbance spectra.

Isolation and Identification of δ-Viniferin. *Apparatus.* The centrifugal partition chromatograph apparatus (FCPC, Kromaton) consisted of a centrifuge with a column of 200 mL capacity. The solvents were pumped into the column rotating at 1000 rpm with HPLC pumps (Gilson, model 321) at a flow-rate of 3 mL/min. Fractions were collected with a fraction collector (Gilson, model FC 204).

Solvents. Solvents were purchased in HPLC-grade quality or redistilled before use. The solvent system for centrifugal partition chromatography was H_2O/EtOH/hexane/EtOAc in the ratio 3/3/4/5 (v/v) (*20*, *26*). The resulting lower layer was used as the stationary phase for the ascendant mode.

Preparation and Fractionation of Red Wine Extract. Red wine (1.4 L) from Brazil (Merlot 2002, Giacomin commercial) was extracted as previously reported (*20*). Briefly, it was concentrated in vacuo and extracted four times with EtOAc. The concentrated EtOAc residue was then chromatographed over a 1.5 × 60 cm cation-exchange resin column (DOWEX, Sigma), rinsed with distilled water (4 L), and eluted with 75% (v/v) aqueous MeOH (2 L) to yield 0.67 g of solid powder after lyophilization. For further fractionation, this residue was submitted to centrifugal partition chromatography (CPC). The ascendant mode yielded five major fractions (A−E), and the descendant mode yielded a single fraction (0.490 g). Monitoring of the collected fractions was achieved by TLC on Polygram silica gel 0.2 mm with fluorescent indicator UV_{254} (Macherey-Nagel) in the mixture $CHCl_3$/MeOH/HCOOH, 85/15/3 (v/v). Visualization of TLC plates was done by spraying anisaldehyde reagent (mixture of 9:1 (v/v) ethanol/acidified *p*-anisaldehyde reagent (*27*).

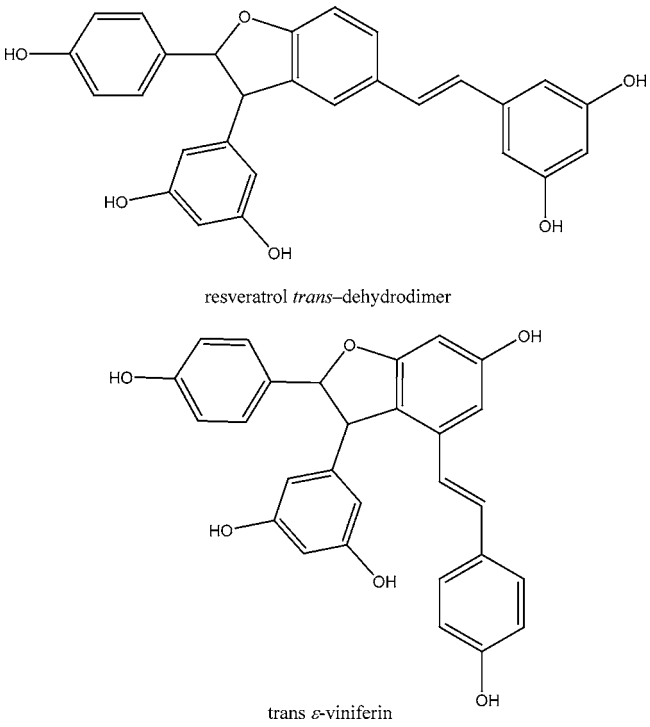

Figure 1. Structures of *trans*-δ-viniferin (trans-dehydrodimer) and ϵ-viniferin.

Final purification of fraction C was achieved using semipreparative HPLC (Varian, model 210) with a 4 × 250 mm Ultrasep RP18 reverse phase column (4 μm) (Bischoff) at room temperature using the solvents H_2O/TFA, 97.5/2.5 (v/v) (A) and ACN/solvent A, 80/20 (v/v) (B) with the gradient system as follows: 30% B (0−10 min), 30−50% B (10−30 min), 50% B (30−40 min), 50−100% B (40−41 min), 100% B (41−46 min), 100−30% B (46−48 min), 30% B (48−53 min). Detection was done at 286 and 306 nm, with a UV−vis detector (Varian, model 345).

Isolation and Identification of *trans*-δ-Viniferin. *trans*-δ-Viniferin (**Figure 1**) was isolated from fraction C by HPLC as a peak collected at t_R = 34 min. It was identified by comparison of its observed ^1H and ^{13}C NMR data with literature values (*28*−*30*). NMR spectra were recorded at 303 K in the Fourier transform mode at 500.13 MHz on a Bruker AMX 500 spectrometer equipped with a broad band 20-mm probe, using a spectral width of 20 ppm and TMS as internal standard. Chemical shifts were expressed as ppm relative to the CD_3OD (3.3 ppm) resonance.

The ^1H NMR spectrum of the compound was characterized by the presence of two vinyl proton signals with a *trans*-geometry. The 1D and 2D NMR data showed the presence of four aromatic rings, two olefenic protons, and an ether ring. The 2D COSY, HMQC, and HMBC correlations permitted determination of the chemical structure of the benzenic rings and their relative position to the ether ring. Furthermore, all NMR data were found to be in full agreement with the structure of the reported *trans*-dehydrodimer (*28*−*30*). **Figure 2** shows the 2D-HMBC spectrum of the trans-dehydrodimer (δ-viniferin) obtained after isolation from red wine.

HPLC Analysis. Separation and quantification of stilbenes (*trans*-resveratrol, *cis*-resveratrol, *trans*-astringin, *trans*-piceid, and ϵ-viniferin

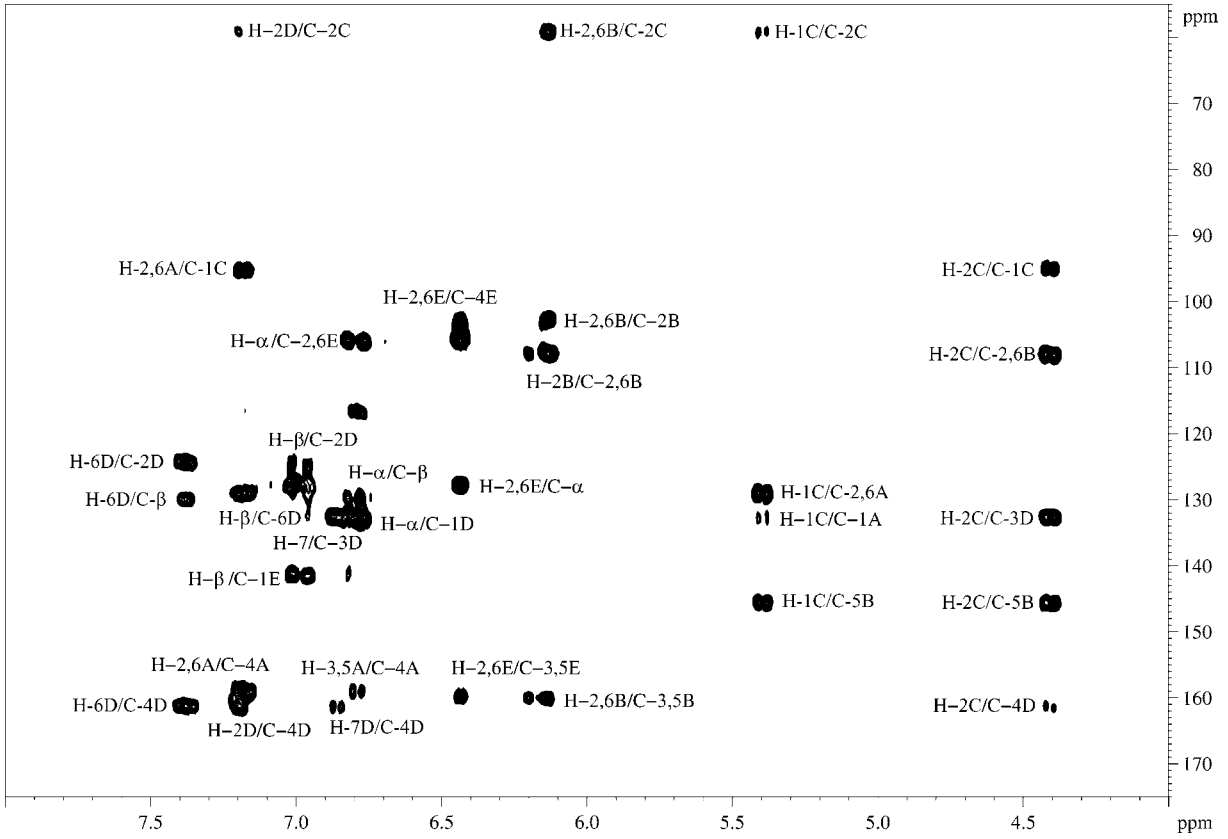

Figure 2. 2D-HMBC spectrum of the trans-dehydrodimer (δ-viniferin) obtained after isolation from red wine.

Table 1. Analytical Characteristics for the Determination of Stilbenes in Wines Samples by HPLC–UV

stilbene	detection limit,[a] ng	calibration range,[b] mg/L	*r*	accuracy,[c] recovery %	precision,[d] RSD%	t_R, retention time, min
trans-astringin	8	0.0–50	0.9993	99.1±3.8	1.5–3.4	34.515
trans-piceid	5	0.0–50	0.9991	99.5±2.5	1.3–3.3	40.230
trans-resveratrol	5	0.0–50	0.9992	99.0±3.9	1.8–2.6	48.957
cis-resveratrol	8	0.0–50	0.9995	99.5±3.5	1.3–3.1	49.332
ϵ-viniferin	8	0.0–50	0.9993	99.1±3.8	1.8–2.9	50.395
δ-viniferin	8	0.0–50	0.9990	99.4±2.7	1.8–3.8	52.223

[a] Detection limit calculated according to IUPAC rules (25 μL). [b] Calibration range in mg/L and coefficient of correlation (*r*) obtained for 5 points. [c] Mean value SD of determinations in two different samples. [d] Relative standard deviation, RSD (%), of six determinations in five different samples.

and δ-viniferin) in wines were carried out by HPLC. A Hewlett-Packard Model 1090 with three low-pressure pumps and a diode array detector coupled to a Hewlett-Packard Chem Station was used for solvent delivery and detection. A Hewlett-Packard column packed with Nucleosil 100 C18 (250 × 4 mm, 5 μm particle size) thermostated at 30 °C was used as stationary phase with a flow rate of 0.5 mL/min. The solvents used for the separation were as follows: solvent A, acetic acid in H_2O, pH 2.4; solvent B, 20% phase A with 80% acetonitrile. Separation was performed with the gradient previously described (*9*). A 25 μL amount of wine was injected into the HPLC system after filtration on a Millipore membrane 0.45 μm. After each analysis, the column was reequilibrated with phase A for 10 min. Detection was carried out at 280, 286, 306, and 321 nm. Measurements were carried out in duplicate. Analytical characteristics of stilbenes determinations are given in **Table 1**.

Wine Samples. We analyzed 12 samples of red wine of various varieties (Merlot, Cabernet-Sauvignon, Cabernet-Franc, Tannat) from several Brazilian wineries. The wine samples were from all viticultural areas of Rio Grande Do Sul and of various vintages, i.e., 1999 to 2003 (**Table 2**). All wines analyzed are frequently consumed in Brazil.

RESULTS AND DISCUSSION

Stilbenes Levels in Wines. Each stilbene compound was determined in wine samples with authentic stilbenes standards (**Table 1**), and results of stilbenes levels in brazilian wines are given in **Table 2**. In this study, levels of δ-viniferin are reported in several red wines for the first time. Concentrations ranged from 0 mg/L to 22 mg/L, with a mean value of 6.4 mg/L. Substantial concentration of δ-viniferin (22 mg/L) was found in one sample of Rio Grande dol Sul (Merlot Giacomin wine, vintage 2002). This red wine was enriched in phenolics by a winemaking technique consisting in crushing the grapes with must, seeds, and skins. In addition, the fermentation process included a long maceration step (15 days) with an increase of temperature to 32 °C. These findings are interesting since such oenological practices may enhance the quantity of stilbenes (particularly resveratrol oligomers) available in the human diet (*31*). The comparison of δ-viniferin levels between several varieties (**Figure 3**) indicated that Merlot contained 10 mg/L δ-viniferin whereas Cabernet-Sauvignon contained only 2 mg/L

Table 2. Levels of Stilbenes in Brazilian Wines (mg/L)

wines	CPT[a]	*trans*-astringin	ϵ-viniferin	*trans*-piceid	*cis*-resveratrol	*trans*-resveratrol	δ-viniferin	total stilbenes[c]
Cabernet-sauvignon 2001	1675	ND[b]	1.59	7.43	7.02	2.06	ND	18.1
Cabernet franc 2001	1900	25.72	1.85	ND	8.43	2.27	ND	38.27
Merlot 2000	2350	ND	4.32	9.84	7.93	3.04	ND	25.13
Tannat 2000	2250	ND	0.64	ND	1.91	ND	ND	2.55
Cabernet sauvignon 2000	2275	ND	1.23	ND	3.04	ND	ND	4.27
Merlot 1999	2175	23.12	0.19	5.3	12.25	ND	6.44	47.3
Merlot 2002 Giacomin commercial	2750	16.72	3.31	20.0	21.4	5.34	20.75	87.52
Merlot 2002 Giacomin in tank	2825	ND	2.83	19.53	22.99	4.86	22.41	72.62
Cabernet sauvignon 2002 Giacomin	2985	ND	2.19	ND	1.7	2.41	6.06	12.36
Merlot 2002	1375	ND	2.45	5.62	4.9	2	3.82	18.79
Cabernet sauvignon 2003 microwinemaking	1050	ND	0.58	ND	4.16	ND	4.98	9.72
Merlot 2003 microwinemaking	1000	4.35	2.35	18.03	23.23	1.77	12.2	61.93

[a] CPT: Total phenol content (in GAE: gallic acid equivalent). [b] ND: Not detected. [c] Expressed as the sum of each stilbene compound (each stilbene compound was determined with an authentic stilbene standard; see **Table 1**).

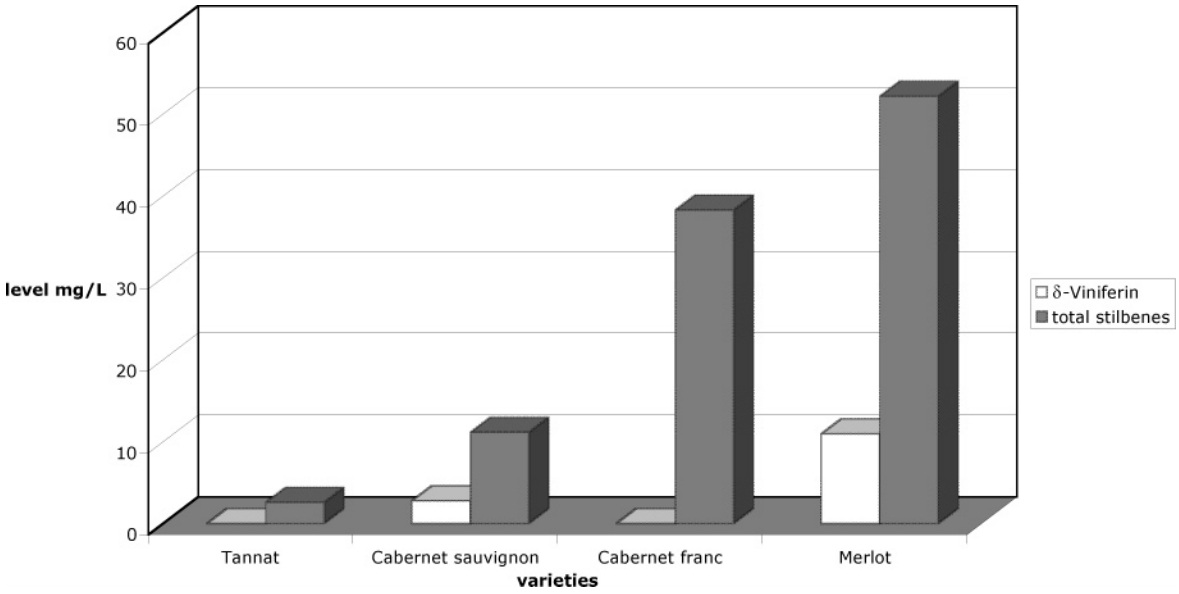

Figure 3. δ-Viniferin and total stilbenes average levels (various vintages wines) by varieties.

Table 3. δ-Viniferin and Total Stilbene Average Levels in Brazilian Vintage Wines (mg/L)

vintages	δ-viniferin	total stilbenes
2000	0	13.84
2001	0	28.19
2002	11.7	43.82

(5 times less). In addition, δ-viniferin could not be detected in traditional wine of Tannat and Cabernet-Franc varieties.

Comparison of δ-viniferin levels between different vintages (**Table 3**) indicated that this compound was detected only in the youngest vintage (2002). It has been shown that viniferins and some others resveratrol dimers are fungal metabolites of resveratrol (*28*). Thus, the occurrence of this compound in wine is certainly due to the oxidation of resveratrol by fungus in infected berries used for vinification. Grapes and wines containing high levels of δ-viniferin could be of health interest since its inhibitory effect on cycloxygenases activities (COX-1 and -2) was reported to be higher than that of *trans*-resveratrol (*17*).

Regarding ϵ-viniferin, its levels ranged from 0.2 to 4.3 mg/L, with an average of 2 mg/L. Wines from Merlot variety had an average of 2.6 mg/L, a value 1.8 times higher than in Cabernet-Sauvignon wines. However, average levels are in agreement with our recent work showing that both red wines and Botrytized sweet white wines contained relatively low levels of ϵ-viniferin (0.1 to 1.6 mg/L) (*21*).

Table 2 shows the levels of the main resveratrol derivatives determined by the HPLC method. *trans*-Astringin was detected in only 33% of wine samples, most of them being Merlot varieties (average 7.4 mg/L). Interestingly, this compound was not found in Tannat and Cabernet-Sauvignon varieties. We found that *trans*-piceid appears to be highest in Merlot wine with a range between 5.3 and 20 mg/L averaging 13 mg/L: 7 times more than in Cabernet-Sauvignon (average 1.9 mg/L). Levels of *trans*-piceid found in Merlot corroborate other previous results with red wines from others countries (*9*), although *trans*-piceid was not found in wines of Tannat and Cabernet-Franc varieties. *trans*-and *cis*-resveratrol levels are reported in **Table 2**. Surprisingly, *cis*-resveratrol levels ranged from 1.7 to 23.2 mg/L, with an average of 10 mg/L, a value 5 times higher than the trans isomer (average 2 mg/L).

The average levels of total stilbenes as a function of grape variety are shown in **Figure 3**: the highest levels were found in Merlot (52 mg/L), followed by Cabernet-Franc (38 mg/L), Cabernet-Sauvignon (11 mg/L), and finally Tannat varieties (only 2 mg/L). Regarding the vintages (**Table 3**), the highest levels of total stilbenes were found in the younger vintages

(2002: 43 mg/L and 2001: 28 mg/L), values more than 2 times higher than in wines from vintage 2000. These results could be attributed to recombinations or degradation of stilbenes during wine aging.

Estimation of Stilbenes Intake from Wine. It is important to monitor food and beverages for phenolics antioxidants because wine phenolics appear to have the properties that could reduce heart disease mortality rates observed in moderate wine drinkers. In this study, all the samples analyzed showed significant levels of stilbenes (average 33 mg/L), and the compounds analyzed are recognized as potent antioxidants. For example, it has been reported that *trans*- and *cis*-resveratrol, *trans*- and *cis*-piceid, and *trans*-astringin are significantly active molecules against LDL oxidation in vitro (*25*). For this reason, we estimated the possible stilbene intake of the Brazilian population drinking wine regularly, based on the wine consumption data of a regular wine consumption of 160 mL/day/individual (corresponding to the french daily intake, *32*). In this case, the current daily intake can be estimated as 5.3 mg/day/individual. For people drinking only Tannat wines, this value is only 0.4 mg/day/individual, but can reach 8.3 mg/day/individual for people drinking only Merlot wine. The stilbenes daily intake value from Brazilian Merlot wine is 1.7 times higher than that from Portuguese Merlot according to our previous work (*9*). We can therefore assume that Brazilian wines, particularly Merlot varieties, can constitute a significant source of daily intake of stilbenes. Our results indicate that Brazilian red wines contained high levels of resveratrol derivatives (namely *trans*-astringin, as well as *cis*-resveratrol in quantities 5 times higher than its trans isomer), which may contribute to a significant proportion in stilbene dietary intake. Among these, the newly isolated resveratrol dimer δ-viniferin can reach levels up to 20% of total stilbenes in Merlot varieties (average 6.4 mg/L versus 33 mg/L total stilbenes).

The origins of differences in stilbene levels observed between varieties require further studies, such as soil composition, winemaking and conservation process, and environmental conditions (fungal pressure, sun exposition, etc.). It would also be important in the future to investigate the bioavailability of the newly isolated stilbene δ-viniferin.

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