Phenolic Characteristics and Antioxidant Activity of Merlot and Cabernet Sauvignon Wines Increase with Vineyard Altitude in a High-altitude Region

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Altitude, as an important factor in the expression of terroir, may affect wine quality. We evaluated the effect of altitude and its related climatic conditions on the phenolic characteristics and antioxidant activity of red wines made from grapes originating from high-altitude areas. The content of total phenolic compounds, total flavonoids and total anthocyanins increased with altitude in Merlot (ME) and Cabernet Sauvignon (CS) wines. Cabernet Sauvignon wines showed richer tannins with increasing altitude. Merlot and CS wines from higher altitude vineyards, showed a greater antioxidant capacity. Salicylic acid, syringic acid, caffeic acid, (+)-catechin, (−)-epicatechin, and the sum of individual phenolic compounds in the wines increased with altitude based on the results of HPLC. The scores of the sensory evaluation of ME wines increased with higher altitude. The highest score was determined for CS wine originating from 2 608 m. A clear grouping of wines according to grape cultivar and vineyard altitude was observed by principal component analysis. Regression analysis showed that altitude, followed by sunshine hours, made the greatest contribution to differences in the phenolic characteristics and antioxidant activity of red wines at different sites in a high-altitude region.

INTRODUCTION

Environmental factors such as light and field management practices have a combined effect on grapevine physiology and wine quality (Spayd *et al*., 2002; Feng *et al*., 2017). The French term "terroir" is used to define the geographical and environmental origin of grapes grown and harvested during a certain vintage (Deloire *et al*., 2008). This term includes all regional parameters, such as soil type, climate and orography (Douglas *et al*., 2001). Terroir is an important factor affecting grape and wine composition (Roullier-Gall *et al*., 2014).

Altitude is usually considered an indirect control on the environmental conditions affecting plant metabolism (Kumar *et al*., 2006). Therefore, altitude may have a strong effect on climatic conditions that affect grape development and wine quality (Mateus *et al*., 2002). In particular, altitude gradient may reflect the integrated variation in temperature, humidity and solar radiation in a mountainous region with high vertical zonality (Zhang *et al*., 2005).

Phenolic compounds contribute to a wine's organoleptic characteristics, such as mouthfeel and colour, and are associated with anti-cancer and cardiovascular protection and antioxidant activity (Burns *et al*., 2000; Skerget *et al*.,

2004). The phenolic compound concentrations of grapes are affected by time of harvest and grape ripeness levels, thereby affecting the flavour and quality of the resulting wine. Phenolic compound concentrations are also affected by genotypes, viticultural practices and environmental conditions (Downey *et al*., 2006). Variation in phenolic characteristics of wine grapes has often been observed at different altitudes and also has been characterised by regional differences (Mateus *et al*., 2001, 2002; Liang *et al*., 2014). Higher altitudes synchronise with a lower temperature and higher humidity, which may be beneficial to carotenoid accumulation (Oliveira *et al*., 2004). An increase in ultraviolet (UV) intensity, with an increase in altitude, promotes anthocyanin biosynthesis in Malbec grape skin via upregulating the genes encoding phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) (Berli *et al*., 2015).

The effect of vineyard altitude on wine quality has not been reported extensively (Jiang *et al*., 2015; Yue *et al*., 2015). Touriga Nacional wine made from grapes harvested at higher altitudes – ranging from 300 m to 350 m – showed a higher level of anthocyanins (Mateus *et al*., 2002). The

concentrations of 2-methoxy-3-isobutylpyrazine in CS wines were found to be positively correlated with vineyard altitude (Falcão *et al*., 2007). Altitude played a more important role than rainfall and growing degree days on the differences in phenolic composition of Malbec wine produced in Mendoza and California at an altitude of 1 103 m and 190 m, respectively (King *et al*., 2014). However, contrasting results have also shown that total phenolic compounds, flavonoids and flavanols in red wine made from grapes originating from lower altitude vineyards were significantly higher than higher altitude vineyards, although anthocyanin content increased with altitude (Jiang *et al*., 2011). No relationship was observed among α- and β-ionone, β-damascenone content and vineyard altitude in Brazilian CS wines (Falcão *et al*., 2007). Most of the aforementioned vineyards were located at altitudes ranging from 39 m to 1 354 m.

This study aimed to determine the differences in phenolic characteristics and antioxidant activity of red wines produced at different altitudes in the high-altitude region of Hengduan Mountain. The objective of this work was to evaluate the effect of altitude and climatic factors on wine quality.

MATERIALS AND METHODS
Chemicals
Methanol, Folin–Ciocalteu reagent, acetonitrile (HPLC grade) and acetic acid were purchased from Sinopharm Chemical Reagent Co. Ltd. (Beijing, China). Gallic acid, rutin, cyanidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, (+)-catechin, trolox, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and all individual phenolic standards for HPLC analysis were purchased from Sigma-Aldrich Company (MO, USA).

Experimental region and grape management
The experimental region is located in Danba County, situated in the north of the Hengduan Mountains, China. The Hengduan Mountain system includes a complex of ridges and river valleys that rise between the western margin of Yunnan Plateau and the eastern margin of Tibet in China and run approximately north to south. This region is famous for vertical zonality, and hence altitude may make a greater contribution to the terroir of wine from this area. Furthermore, the vineyards are mostly located at altitudes from 2 000 m to 3 000 m.

This wine region is located at the low latitude of 30°N to 31°N and is characterised by a cool climate with a mean annual temperature of 13°C, with substantial differences between day and night temperatures (23°C minimum temperature and 37°C maximum temperature) and, with 600 mm of annual rainfall, less rainfall compared to the Shandong wine regions in China. Vineyards are often established on steep slopes and therefore vines are not covered during the winter months. Two red wine grape cultivars of *Vitis vinifera* L., viz. Cabernet Sauvignon (CS) and Merlot (ME), were selected in 2014. The vineyard sites were spread over three altitudes (2 282 m, 2 435 m and 2 608 m). Cabernet Sauvignon and ME grapevines were planted in a same vineyard at 2 282 m and 2 435 m respectively. One CS vineyard was located at 2 608 m. All grapevines were established in 2006, planted in east–west row orientations and trained to a vertical trellis

with Guyot pruning. The vines were planted with a 2.0 m spacing between rows and a 1.0 m spacing between vines. Shoot thinning was applied to adjust the grape yield of each vineyard to a similar level of about 10 tons per hectare.

Climate data
Climate data were collected from the automatic meteorological stations (TRM-ZS3, Yangguang Company Ltd., Jinzhou, China). One station was positioned in the centre of the vineyard at 2 282 m, 2 435 m and 2 608 m. The average annual values of climatic parameters were calculated based on the daily data.

Winemaking process
Grapes from each vineyard were manually harvested at commercial maturity in 2014. The total soluble solid content of grapes from the ME vineyard at 2 282 m, the CS vineyard at 2 282 m, the ME vineyard at 2 435 m, the CS vineyard at 2 435 m and the CS vineyard at 2 600 m was 20.4°Brix, 21.2°Brix, 20.7°Brix, 21.4°Brix and 21.6°Brix, respectively. The grape bunches were destemmed and crushed with an experimental destemmer-crusher and transferred to 50 L stainless steel containers. Sulphur dioxide (60 mg/L) was added to the must. *Saccharomyces cerevisiae* (RC 212, Lallemand, Danstar Ferment AG, Switzerland) active dry yeast (0.2 g/L) was inoculated to initiate fermentation, which lasted for seven to eight days at 25 to 28°C. Density control and temperature were monitored daily during fermentation. The contents of the tank were homogenised once a day to dissolve the cap into the wine. Wines were transferred to another tank after fermentation for three weeks of cold treatment at 4°C, after which the wines were bottled. Chemical analyses of the wines were carried out two months after bottling. Wines of each sampling vineyard were produced in three replications.

Analyses of general oenological parameters
The general parameters of wines, including alcohol content (AC), titratable acidity (TA), volatile acidity (VA), pH, reducing sugars (RS) and colour intensity (CI) were analysed according to the official methods established by the OIV (2014).

Determination of phenolic content
Total phenolic content (TPC)
The TPC was measured using the Folin–Ciocalteu method (Singleton & Rossi, 1965). In brief, 5.99 mL of distilled water, 0.1 mL of wine sample and 0.2 mL of Folin–Ciocalteu reagent were added to a test tube in sequence. Sodium carbonate solution (2 mL, 10%, w/v) was added to the tube after the contents had been reacting for 5 min. The mixture was placed in a dark room to react at room temperature for 120 min. Absorbance was measured at 765 nm. The results were expressed as gallic acid equivalents (GAE) in mg/L.

Total monomeric anthocyanin content (TMAC)
The TMAC was determined using the pH differential method (Wrolstad, 1976). Wine samples were diluted 20-fold with a buffer at pH 1.0 and pH 4.5, respectively. Absorbance was measured at 510 nm and 700 nm in both the pH 1.0 and pH

4.5 buffers and calculated by the following equation: A = $(A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}$. The TMAC (expressed as cyanidin-3-*O*-glucoside equivalent, C3GE) was calculated using the equation: TMA content (mg/L of C3GE) = (A × MW × DF × 1000)/(ε × 1), where A is the absorbance, MW is the molecular weight of cyanidin-3-*O*-glucoside (449 g mol/L), DF is the dilution factor, and ε is the molar extinction coefficient of cyanidin-3-*O*-glucoside (29 600).

Tannin content

Tannin content was determined by protein precipitation using the method of Harbertson *et al*. (2003). Tannins were precipitated from wines by adding bovine serum albumin solution of 1 g/L, followed by centrifugation, collection and resuspension of the precipitate in an alkaline triethanolamine/sodium dodecyl sulphate buffer. Background absorbance was determined at 510 nm, and tannin absorbance was measured after adding a $FeCl_3$ solution (2.7 g/L). The results for quantification were compared to a (+)-catechin standard and reported as (+)-catechin equivalents (CE) in mg/L.

Content of anthocyanin composition

Wine anthocyanin composition was analysed by using the reversed-phase HPLC method as described by Lamuela-Raventós and Waterhouse (1994), with some modifications. Twenty microlitres of wine samples were directly injected after filtration using 0.45 μm organic filters. The chromatograph system (Shimadzu, Japan) was equipped with a LC-20AT pump, a SIL-20A autosampler, a SPD-M20A diode array detector (Shimadzu, Japan), and a CTO-20A column oven. A SEP-PAK C18 (250 × 4.6 mm i.d., 5 μm) (Waters, USA) was used and the column temperature was 30°C. The detection wavelength was 520 nm. Solvent A was 10% (v/v) formic acid in water and solvent B was acetonitrile:solvent A (80:20, v/v). These solvents were passed through a 0.20 μm filter. The gradient was as follows: 0 to 45 min, 10% to 60% B; 45 to 46 min, 60% to 100%; 46 to 50 min, 100% to 10% B; 50 to 55 min, 10% to 10% B, with a flow rate of 1.0 mL/min. All analyses were performed in three replicates. External calibration was performed using a malvidin-3-*O*-glucoside standard. All other anthocyanins were quantified using this calibration curve and reported as malvidin-3-*O*-glucoside equivalents in mg/L.

HPLC analysis of individual phenolic compounds

The HPLC analysis was performed using a LC-20A HPLC system (Shimadzu, Kyoto, Japan) with a Hibar RT LiChrospher RP-C18 column (250 × 4.0 mm, 5 μm) purchased from Merck, Darmstadt, Germany, based on the method of Cheng (2008). Solvents A and B were composed of 2% (v/v) acetic acid in water and 100% acetonitrile respectively. The following gradient was optimised after preliminary experiments: 0 to 15 min, 3 to 6% B; 15 to 35 min, 6 to 15% B; 35 to 55 min, 15 to 30% B; 55 to 65 min, 30 to 30% B; 65 to 80 min, 30 to 0% B. The column temperature was 30°C, and the flow rate was 1.0 mL/min. The photodiode array detector scanned from 200 nm to 400 nm. The analysis for each sample was performed in triplicate.

The identification of phenolic compounds was based on a comparison of their retention time with those obtained from authentic standards and spiked samples with the standard solutions. Quantification was performed using the external standard method. A six-point calibration curve was used for each standard.

Measurement of antioxidant activity
DPPH free radical elimination activity
The DPPH was determined by the method of Brand-Williams *et al*. (1995). The wine sample and DPPH methanolic solution were mixed and kept in a dark room for 30 min. The absorbance of the reaction mixture was measured at 517 nm. The results were expressed as trolox equivalents (TE) in μmol/L.

Cupric-reducing antioxidant capacity (CUPRAC)
The CUPRAC was assayed based on the method of Apak *et al*. (2004). In brief, wine samples were diluted 10-fold with distilled water. The dilutions were mixed with $CuSO_4$, neocuproine and distilled water. Absorbance was measured at 450 nm after 30 min. The results were expressed as TE in μmol/L.

Sensory evaluation
The sensory profiles of the ME and CS wines originating from different altitudes were analysed in the wine sensory laboratory, College of Oenology, Northwest A&F University. The sensory panel was composed of 15 trained wine panellists (eight males and seven females, 21 to 45 years of age) who major in oenology. The panellists included graduate students and teachers and were chosen according to their experience in wine sensory analysis training programmes. All had attended a wine-tasting training class once a week for three months.

The evaluation consisted of describing the visual aspect, aroma, taste and harmony, which accounted for 15, 30, 44 and 11 scores respectively (Li, 2006). Wine samples (30 mL) were presented in standard ISO wine glasses (ISO 3591:1977) covered with plastic lids. Wines were coded randomly and were presented to the panel arbitrarily. Wine samples were evaluated in triplicate. Water and unsalted crackers were provided as palate cleansers and all samples were expectorated.

Statistical analysis
All the analyses were performed in triplicate. Data were expressed as the mean ± standard deviation values and analysed by one-way ANOVA with a Tukey post-hoc test at 5% using SPSS 19.0 software (SPSS, Chicago, IL). A two-tailed Pearson's correlation test was conducted to determine the correlations between the phenolic content and antioxidant activity of the wines. Principal component analysis (PCA) was used to explore the contribution of each parameter of phenolic characteristics and antioxidant activity to the clustering among the wines originating from different altitudes. The least absolute shrinkage and selection operator (LASSO) method was performed using Matlab R2010b software (Mathworks, Natick, MA) for regression analysis between environmental factors and phenolic characteristics and the antioxidant activity of the wines.

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RESULTS AND DISCUSSION

Soil properties and climate parameters of different altitudes and vineyards

The terroir, consisting mainly of climate, soil and geomorphology properties, was one of the factors affecting wine characteristics. The soil properties of the sampling vineyards at different altitudes are presented in Table 1. The same soil texture and similar chemical properties were established in the different soil samples originating from the different vineyards. Table 2 shows the main annual climate parameters in 2014. Mean annual temperature, annual maximum temperature, annual minimum temperature, effective accumulative temperature and active accumulated temperature parameters decreased with increasing altitude. Average temperature differences between daytime and night time increased as altitude increased, up to 13.0°C at 2 608 m. An increase in rainfall and sunshine hours was concomitant with an increase in altitude. The maximum wind speed and minimum relative humidity were determined at 2 435 m.

Oenological parameters

The general composition of five wine samples at three different altitudes are described in Table 3. No significant differences in the alcohol content (AC) of ME and CS wines were detected with increasing altitude. Higher AC was determined for CS wines compared to ME wines at the same altitude. The colour intensity (CI) of the wines increased with an increase in altitude and differences in CI were also measured between ME and CS wines. The CS wines had a higher CI value than the ME wines at 2 282 m and 2 435 m. The CI of the wine was strongly related to the anthocyanin content, which varied by grape cultivar and was strongly affected by climatic conditions (Buzo *et al.*, 2014). The increased radiation, especially UV radiation, usually observed at higher altitudes would benefit the synthesis of anthocyanins in grape skin (Zhang *et al.*, 2012; Berli *et al.*, 2015), which could be ascribed to the higher CI observed in wines at higher altitudes.

TABLE 1

Physicochemical properties of soil in the five sampling vineyards.

Vineyard	Soil texture	pH	Organic matter (g/kg)	Water-soluble Ca (mg/kg)	Water-soluble Mg (mg/kg)	Available N (mg/kg)	Available P (mg/kg)	Available K (mg/kg)
ME2282	sandstone	8.09	21.79	108.51	16.61	49.79	8.80	123.69
CS2282	sandstone	8.15	20.02	105.24	18.93	52.88	9.70	145.69
ME2435	sandstone	7.95	35.26	192.90	18.15	52.45	15.30	158.25
CS2435	sandstone	7.88	39.83	134.79	17.21	41.81	15.80	143.24
CS2608	sandstone	8.24	23.89	162.04	21.86	47.62	14.50	223.89

ME2282, CS2282, ME2435, CS2435 and CS2608 indicate the Merlot vineyards at 2 282 m, the Cabernet Sauvignon vineyards at 2 282 m, the Merlot vineyards at 2 435 m, the Cabernet Sauvignon vineyards at 2 435 m, and the Cabernet Sauvignon vineyards at 2 608 m, respectively.

TABLE 2

Annual climate parameters at different altitudes in 2014.

Altitude (m)	MAT (°C)	AHT (°C)	ALT (°C)	ATD (°C)	RH (%)	R (mm)	SH (h)	WS (m/s)	EAT (°C)	AAT (°C)
2282	14.0	37.9	-5.3	12.6	53.6	618.9	1967.6	1.9	2192	4882
2435	13.2	37.5	-5.7	12.7	52.7	621.2	1996.5	3.4	2121	4761
2608	12.3	35.4	-6.3	13.0	53.5	767.9	2067.9	3.0	2068	4688

MAT: mean annual temperature, AHT: annual highest temperature, ALT: annual lowest temperature, ATD: average temperature difference between daytime and night time, RH: relative humidity, R: rainfall, SH: sunshine hours, WS: wind speed, EAT: effective accumulative temperature, AAT: active accumulated temperature.

TABLE 3

Oenological parameters of wines from different altitudes and cultivars.

Wine	AC (%, v/v)	TA (tartaric acid, g/L)	VA (acetic acid, g/L)	pH	RS (g/L)	CI (AU)
ME2282	11.51 ± 0.35b	5.38 ± 0.34c	0.51 ± 0.03a	3.48 ± 0.15a	2.02 ± 0.05a	7.9 ± 0.5c
CS2282	12.04 ± 0.24a	5.32 ± 0.32c	0.52 ± 0.04a	3.53 ± 0.18a	1.77 ± 0.02b	9.7 ± 0.7b
ME2435	11.72 ± 0.25b	6.44 ± 0.29a	0.53 ± 0.02a	3.30 ± 0.21b	1.68 ± 0.04b	9.4 ± 0.6b
CS2435	12.13 ± 0.28a	5.49 ± 0.23b	0.48 ± 0.03a	3.39 ± 0.12b	1.62 ± 0.02b	10.3 ± 0.6a
CS2608	12.25 ± 0.31a	5.63 ± 0.37b	0.51 ± 0.06a	3.36 ± 0.15b	1.50 ± 0.03c	10.7 ± 0.5a

AC: alcohol content, TA: titratable acidity, VA: volatile acidity, RS: reducing sugars, CI: colour intensity.
ME2282, CS2282, ME2435, CS2435 and CS2608 refer to Merlot wine at 2 282 m, Cabernet Sauvignon wine at 2 282 m, Merlot wine at 2 435 m, Cabernet Sauvignon wine at 2 435 m, and Cabernet Sauvignon wine at 2 608 m, respectively.

Phenolic content and antioxidant capacity of wines at different altitudes

Phenolic compound concentrations are one of the most important factors affecting the wine quality because of their contribution to colour, mouthfeel and the multiple biological effects of antioxidant activity. The phenolic content and antioxidant activity of the wines at different altitudes are presented in Table 4. The total phenolic content (TPC) of ME and CS wines increased significantly from 2 282 m to 2 435 m. The CS wine made from grapes originating at 2 608 m showed the highest TPC value, which was significantly higher than that of the other wine samples.

The total monomeric anthocyanin content (TMAC) of the wine increased markedly with altitude in the ME and CS wines produced in the higher altitude region. This was in agreement with the results for Touriga Francesca wines, in which anthocyanin content increased with increasing altitude from 150 m to 250 m (Mateus *et al.*, 2002), as observed for the CS grapes (Liang *et al.*, 2014). Anthocyanins originally synthesised in the skin of red grapes after véraison are responsible for the colour and stability of wine. Sunlight, especially UV radiation, is one of the major environmental factors generally suggested to affect anthocyanin biosynthesis by prolonging the activity of phenylalanine ammonia lyase (PAL), which is the key enzyme in the flavonoid biosynthesis pathway (Berli *et al.*, 2015). Hence, the exposure of grape bunches to sunlight could increase the accumulation of total anthocyanins in the grape skin (Spayd *et al.*, 2002). The sunshine hours usually increased with altitude, as shown in Table 2. Furthermore, the intensity of UV radiation would increase with increasing altitude in the mountainous region (Zhang *et al.*, 2005). The CS wines showed a higher anthocyanin content than ME wines at 2 282 m and 2 435 m, respectively. The anthocyanins in the wine were extracted from grape berries during fermentation and maceration. Varietal genetic characteristics and the extended harvest time of CS grapes might be beneficial to anthocyanin accumulation and contribute to their high content in wine under the same maceration and fermentation conditions (Jiang & Zhang, 2012). Furthermore, the average temperature difference between daytime and night time was found to increase from 2 282 m to 2 608 m in the present study (Table 2). The temperature difference between daytime and night time has an essential impact on grape sugar accumulation, which could be coupled with the conversion of anthocyanidin into anthocyanins in grapes (Liang *et al.*, 2014). Therefore, grapes at higher altitudes subjected to major differences in temperature between day and night could explain the high anthocyanin content observed in the wines (Yamane *et al.*, 2006).

Tannins in red wine are mostly composed of proanthocyanidins, also known as condensed tannins. Condensed tannins are polymeric compounds made up of flavan-3-ol subunits, which contribute mainly to the bitterness and astringency of wine, and to colour stability in combination with anthocyanins (Vidal *et al.*, 2002). No significant variation in tannin content was observed for the ME wines between 2 282 m and 2 435 m. However, the tannin content increased with altitude for CS wines, up to a maximum of 975 mg/L CE at 2 608 m. Moreover, there were significant differences between ME and CS wines originating from 2 435 m. The tannins in wine are extracted primarily from the seeds and skins during the vinification process and their content in wine depends mainly on the total content in the skins and seeds. Tannin content also varies between grape cultivars (Mateus *et al.*, 2001). Hence, the differences in tannin content between CS and ME wines could be attributed to differences in grape cultivar and altitude.

The chemical diversity of antioxidants makes it difficult to separate and quantify antioxidants in a wine matrix. Therefore, the total level of antioxidant activity is usually measured. A single antioxidant test for the examination of multifunctional antioxidants is insufficient; therefore, more than one method is used to provide adequate information on the antioxidant properties of phenolics (Lutz *et al.*, 2011). To characterise the antioxidant potential of wines made from grapes harvested at different altitudes, two antioxidant assays, viz. DPPH and CUPRAC, were performed. The same variation in altitude was determined for the two antioxidant parameters in this study. The DPPH and CUPRAC values increased significantly with altitude for the ME and CS wines. A maximum value of 5 184 µmol/L TE for DPPH and 16 886 µmol/L TE for CUPRAC was determined in the CS wine at 2 608 m. However, there was no clear difference between the antioxidant parameters of ME and CS wines at 2 282 m and 2 435 m.

TABLE 4

Phenolic content and antioxidant activity of wines from different altitudes.

Wine	TPC (mg/L, GAE)	TMAC (mg/L, C3GE)	Tannins (mg/L, CE)	DPPH (µmol/L, TE)	CUPRAC (µmol/L, TE)
ME2282	1 003 ± 10c	258 ± 3e	842 ± 15c	4 413 ± 50c	13 102 ± 209c
CS2282	1 009 ± 9c	323 ± 7c	861 ± 15c	4 499 ± 111c	13 158 ± 97c
ME2435	1 032 ± 5b	291 ± 6d	859 ± 14c	4 807 ± 121b	15 470 ± 157b
CS2435	1 045 ± 5b	374 ± 5b	917 ± 11b	4 792 ± 135b	15 767 ± 258b
CS2608	1 076 ± 10a	455 ± 5a	975 ± 11a	5 184 ± 35a	16 886 ± 91a

The data were mean values of triplicate samples. Different letters within rows indicate statistical differences according to ANOVA with a Tukey post-hoc test at 5%.
TPC: Total phenolic content; TMAC: total monomeric anthocyanin content; C3GE: cyanidin-3-*O*-glucoside equivalent; DPPH: 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity; CUPRAC: cupric-reducing antioxidant capacity.
ME2282, CS2282, ME2435, CS2435 and CS2608 refer to Merlot wine at 2 282 m, Cabernet Sauvignon wine at 2 282 m, Merlot wine at 2 435 m, Cabernet Sauvignon wine at 2 435 m, and Cabernet Sauvignon wine at 2 608 m, respectively.

Phenolics are characterised by polyphenolic structures containing numerous double bonds and hydroxyl groups that can donate electrons through resonance to stabilise free radicals, and thus act as powerful antioxidants to protect plants against oxidative stress (Machlin & Bendich, 1987). Hence, a positive correlation may be found between the phenolic composition and the antioxidant activity of wines. The correlation coefficient between the phenolic content and the antioxidant activity of wines in the high-altitude region is presented in Table 5. The TPC, TMAC and tannin contents observed were positively and significantly correlated with the results of DPPH and CUPRAC. This is in agreement with the findings of previous research (Jiang *et al*., 2011; Meng *et al*., 2012).

Content of wine anthocyanin composition
Five monomeric anthocyanins, namely delphinidin-3-*O*-monoglucoside (Dp), cyanidin-3-*O*-monoglucoside (Cy), petunidin-3-*O*-monoglucoside (Pt), peonidin-3-*O*-monoglucoside (Pn), and malvidin-3-*O*-monoglucoside (Mv), were determined (Table 6). Mv was the most abundant in the CS and ME wines. This is in agreement with previous reports by Bouzas-Cid *et al*. (2016) and González-Neve *et al*. (2016). Furthermore, significant differences in Mv content were observed among the wines originating from different altitudes and grape cultivars. ME wines originating from 2 435 m showed a higher content of five monomeric anthocyanins compared to the wines originating from 2 282 m. As for the CS wines, the Dp, Cy and Mv content

increased significantly with increasing altitudes. There was no notable difference for Pt and Pn content between CS wines originating from 2 282 m and 2 435 m.

Anthocyanins in *Vitis vinifera* L. cultivars are glycosylated derivatives (He *et al*., 2012). The monomeric anthocyanins in red wines originate mainly from the grape skins. Environmental factors could affect the biosynthesis and accumulation of each anthocyanin in the skin (Liang *et al*., 2014). A higher altitude generally corresponds to more intense sunlight, lower temperature, greater temperature difference between day and night, and more extreme environmental conditions (Mateus *et al*., 2001), all of which are important factors affecting the production of anthocyanins. Furthermore, the effects of altitude on the different monomeric anthocyanins in CS grapes were also different in high-altitude areas (Xing *et al*., 2015).

Changes in concentration of individual phenolic compounds in wine originating from different altitudes
Individual phenolic compounds in wine were measured (Table 7) to further understand the phenolic characteristics of the ME and CS wines produced at different altitudes. An increase in the sum of individual phenolic compound concentrations was shown with an increase in altitude for ME and CS wines. There also was a clear difference in phenolic compound concentrations between ME and CS wines. CS wines originating from 2 282 m and 2 435 m showed a higher total content of individual phenolic compounds compared to ME wines. The gallic acid and salicylic acid content contributed mainly to the sum of individual non-flavonoid phenolics in the wine from the two cultivars at any altitude. Gallic acid is one of the hydroxybenzoic acids present in wine and usually shows the highest concentration. It not only originates from the grape, but is also formed through the hydrolysis of hydrolysable and condensed tannins, i.e. gallic acid esters of flavan-3-ols (Dewick & Haslam, 1969). However, no significant changes in gallic acid concentration were determined in wine from the different altitudes and grape cultivars in the current study. Salicylic acid in the wine increased markedly with altitude for both the ME and CS wines; CS wines originating from 2 282 m and 2 435 m showed a higher content compared to ME wines. Salicylic acid is a secondary metabolite in plants and is stimulated

TABLE 5
Correlation coefficients between phenolic content and antioxidant activity of wines.

	DPPH	**CUPRAC**
TPC	0.874 [**]	0.922 [**]
TMAC	0.823 [**]	0.783 [**]
Tannins	0.825 [**]	0.825 [**]
DPPH	1.000	0.931 [**]
CUPRAC	0.931 [**]	1.000

[**] $p \leq 0.01$

TABLE 6
Anthocyanin composition (mg/L, malvidin-3-*O*-glucoside) of wines from different altitudes.

Wine	Dp	Cy	Pt	Pn	Mv	Total
ME2282	4.65 ± 0.21d	0.35 ± 0.02d	9.20 ± 0.41d	5.94 ± 0.27c	96.32 ± 4.32e	116.48 ± 5.22e
CS2282	5.71 ± 0.42c	0.48 ± 0.04c	12.30 ± 0.90bc	7.52 ± 0.55b	124.35 ± 9.08c	150.37 ± 10.98c
ME2435	6.01 ± 0.31bc	0.68 ± 0.04b	11.17 ± 0.58c	7.68 ± 0.40b	114.12 ± 5.97d	139.68 ± 7.30d
CS2435	6.89 ± 0.42b	0.52 ± 0.03c	13.60 ± 0.82b	8.78 ± 0.53b	142.39 ± 8.63b	172.17 ± 10.43b
CS2608	8.62 ± 0.43a	0.98 ± 0.05a	16.03 ± 0.79a	11.02 ± 0.54a	163.72 ± 8.08a	200.39 ± 9.89a

The data were mean values of triplicate samples; different letters within rows indicate statistical differences according to ANOVA with a Tukey post-hoc test at 5%.
Dp: delphindin-3-*O*-monoglucoside; Cy: cyanidin-3-*O*-monoglucoside; Pt: petunidin-3-*O*-monoglucoside; Pn: peonidin-3-*O*-monoglucoside; Mv: malvidin-3-*O*-monoglucoside.
ME2282, CS2282, ME2435, CS2435 and CS2608 refer to Merlot wine at 2 282 m, Cabernet Sauvignon wine at 2 282 m, Merlot wine at 2 435 m, Cabernet Sauvignon wine at 2 435 m and Cabernet Sauvignon wine at 2 608 m, respectively.

TABLE 7

Content of individual phenolic compounds (mg/L) of wines from different altitudes and cultivars.

Phenolic compounds	Wine				
	ME2282	CS2282	ME2435	CS2435	CS2608
Nonflavonoid					
Hydroxybenzoic acids	38.23 (27.85%)	42.36 (27.85%)	48.92 (28.46%)	37.74 (24.65%)	40.01 (23.69%)
Gallic acid	17.59 ± 0.01a	17.80 ± 0.03a	17.52 ± 0.01a	17.73 ± 0.03a	17.59 ± 0.01a
Benzoic acid	2.18 ± 0.03b	2.14 ± 0.02b	2.30 ± 0.02a	2.25 ± 0.04a	2.29 ± 0.02a
Vanillic acid	2.55 ± 0.08b	1.03 ± 0.06c	5.12 ± 0.26a	1.58 ± 0.12c	4.82 ± 0.40a
Syringic acid	1.52 ± 0.26e	2.32 ± 0.14d	4.04 ± 0.22c	8.71 ± 0.28b	9.61 ± 0.34a
p-Coumaric acid	5.18 ± 0.03c	5.47 ± 0.03b	5.24 ± 0.02c	5.47 ± 0.06b	5.69 ± 0.04a
Salicylic acid	7.71 ± 0.21e	10.64 ± 0.40c	9.20 ± 0.18d	13.59 ± 0.65b	14.70 ± 0.48a
Hydroxycinnamic acids	7.91 (5.76%)	9.22 (6.06%)	8.12 (5.26%)	9.47 (6.18%)	10.28 (6.09%)
Chlorogenic acid	nd	nd	nd	nd	nd
Caffeic acid	4.60 ± 0.05d	4.70 ± 0.03cd	4.87 ± 0.11c	5.15 ± 0.05b	5.35 ± 0.08a
Ferulic acid	3.31 ± 0.04c	4.52 ± 0.07b	3.25 ± 0.03c	4.31 ± 0.19b	4.94 ± 0.08a
Stilbenes	1.88 (1.37%)	1.67 (1.10%)	1.67 (1.08%)	2.38 (1.55%)	3.93 (2.32%)
trans-Resveratrol	1.88 ± 0.04c	1.67 ± 0.07c	1.67 ± 0.04c	2.38 ± 0.14b	3.93 ± 0.08a
Benzopyrone	1.54 (1.22%)	2.97 (1.96%)	1.91 (1.24%)	1.57 (1.03%)	2.18 (1.29%)
Coumarin	1.54 ± 0.30c	2.97 ± 0.23a	1.91 ± 0.10bc	1.57 ± 0.03c	2.18 ± 0.17b
Sum of individual nonflavonoid	49.56 (36.11%)	56.23 (36.97%)	60.63 (39.28%)	51.16 (33.41%)	56.41 (33.40%)
Flavonoid					
(+)-Catechin	32.22 ± 0.20d	34.29 ± 0.20c	33.82 ± 0.30c	38.40 ± 0.33b	40.75 ± 0.32a
(−)-Epicatechin	16.19 ± 0.16e	18.15 ± 0.12c	17.60 ± 0.26d	18.95 ± 0.18b	23.22 ± 0.15a
Flavan-3-ols	48.41 (35.27%)	52.44 (34.48%)	51.42 (33.32%)	57.35 (37.45%)	63.97 (37.88%)
Rutin	12.45 ± 0.19d	17.34 ± 0.21b	15.76 ± 0.19c	17.40 ± 0.33b	22.83 ± 0.40a
Morin	nd	nd	nd	nd	nd
Quercetin	21.46 ± 0.15b	20.39 ± 0.03d	21.39 ± 0.07b	20.71 ± 0.09c	21.98 ± 0.11a
Kaempferol	5.37 ± 0.05a	5.37 ± 0.03ab	5.14 ± 0.02d	5.25 ± 0.05c	5.27 ± 0.04bc
Hesperetin	nd	nd	nd	nd	nd
Flavonols	39.28 (28.62%)	43.41 (28.54%)	42.28 (27.40%)	44.63 (29.14%)	48.49 (28.72%)
Sum of individual flavonoid	87.70 (63.89%)	95.85 (63.03%)	93.70 (60.72%)	101.97 (66.59%)	112.46 (66.60%)
Sum of individual phenolics	135.76 ± 1.40d	148.81 ± 1.14c	148.83 ± 1.09c	163.46 ± 2.03b	185.16 ± 1.89a

The data were mean values of triplicate samples; different letters within lines indicate statistical differences according to ANOVA with a Tukey post-hoc test at 5%; nd: not detected.

ME2282, CS2282, ME2435, CS2435 and CS2608 refer to Merlot wine at 2 282 m, Cabernet Sauvignon wine at 2 282 m, Merlot wine at 2 435 m, Cabernet Sauvignon wine at 2 435 m and Cabernet Sauvignon wine at 2 608 m, respectively.

by certain abiotic stresses such as increased UV radiation (Cohen & Kennedy, 2010). Hence, grapes at higher altitude might synthesise more salicylic acid for protection against UV radiation, which could explain the higher salicylic acid content observed in wine produced from grapes at the higher altitude sites. The syringic acid content in ME and CS wines increased with increasing altitude, up to a maximum of 9.61 mg/L for CS wine originating from 2 608 m. Two hydroxycinnamic acids were detected in the wines. A higher caffeic acid content was found in ME and CS wines with increasing altitude. However, no significant differences in ferulic acid content were found between wines originating

from 2 282 m and 2 435 m. The *trans*-resveratrol content in the CS wines increased with increasing altitude, although there were no differences between ME wines originating from 2 282 m to 2 435 m. This is in agreement with the results from CS wines produced in the Loess Plateau region (Jiang *et al.*, 2015).

The sum of individual flavonoids contributed the most of the total phenolic content at any altitude, ranging from 60.72% to 66.60%. Catechin, which is one of the common subunits of proanthocyanidins, was the predominant phenolic monomer compound in ME and CS wines. It increased significantly with an increase in altitude for the two grape

cultivars, up to a concentration of 40.75 mg/L for CS wine at 2 608 m. Epicatechin, the epimer of (+)-catechin, showed the same trend with altitude for both wines. Furthermore, CS wines showed higher (+)-catechin and (-)-epicatechin contents than ME wines from the same altitude. This is in agreement with work by Li *et al.* (2011), who found that CS wines from different regions in China were higher in (+)-catechin and (-)-epicatechin contents when made from grapes grown at an altitude of 1 214 m compared with those grown at 1 036 m in the western regions. The terroir effect might explain the differences because of the great variation in climate, for example the temperature difference between daytime and night time and the increased annual sunshine between these two regions (Liang *et al.*, 2014). However, the sampled vineyards for the present study were located in the same wine-producing region with similar soil properties. Hence, the flavan-3-ols in grapes, including (+)-catechin and (-)-epicatechin, might be very sensitive to environmental factors, even though the climatic parameters varied little among the three altitudes.

Flavonols are generally considered to act as UV protectants and free-radical scavengers (Downey *et al.*, 2003). Hence, the flavonol content of grapes increases with an increase in sun/light exposure in the fruiting zone (Spayd *et al.*, 2002). Quercetin derivatives such as rutin, including quercetin and kaempferol, comprised most of the flavonol content of ME and CS wines at the different altitudes. The rutin content of ME wines increased significantly from 2 282 m to 2 435 m, but no increase in the same compounds was observed in CS wine. However, the rutin content of CS wines at 2 608 m was notably higher than that at other altitudes. The quercetin content showed no significant increase with altitude in ME wines. The quercetin content in CS wines increased from 20.39 mg/L to a maximum of 21.98 mg/L with an increase in altitude from 2 282 m to 2 608 m. The quercetin content in grape berries usually increases in response to increased exposure to UV radiation in the fruiting zone (Azuma *et al.*, 2012), resulting in high levels of quercetin in wines made from grapes originating from higher altitude vineyards.

Projection of wines on the first two principal components responsible for 70.82% showed a separation mainly according to grape cultivar and vineyard altitude (Fig. 1A). Furthermore, wines from the three altitudes were clearly separated from each other. Cabernet Sauvignon wines showed more separation between the three altitudes than ME wines. Merlot wines at 2 282 m and 2 435 m were located in the positive part of PC2, while CS wines at these two altitudes formed two groups in the negative part of PC2. Cabernet Sauvignon wines at 2 608 m grouped together and were located in the upper positive part of PC1, separate from all the other wines.

The PCA results of the variables used for the characterisation of the wine samples are displayed in the first two principal components and are presented in Fig. 1B. It is notable that component 1 was responsible for 70.82% of the cumulative variance and was mainly associated with the phenolic content and antioxidant capacity and prevailed in the positive part of PC1. Component 2 was responsible for 17.34% of the cumulative variance and was associated with kaempferol and is present in the negative part of PC1. Anthocyanins and tannins are two of the most important flavonoid groups in wine because of their contribution to wine colour, astringency and ageing potential. The TMAC, monomeric anthocyanins and condensed tannin monomers, including (+)-catechin and (−)-epicatechin, were located in the positive part of PC1 and showed a high factor loading with PC1. Hence, wines located in the right half of the diagram were associated with anthocyanins and tannins and those in the left half were related to both varietal character and altitude.

Sensory analysis

The average scores of each wine originating from different altitudes are presented in the global sensory evaluation (Table 8). Higher scores indicated improved attributes of the wine. Cabernet Sauvignon wines originating from 2 282 m had higher scores than ME wines with respect to taste quality and harmony indicators. Cabernet Sauvignon wines originating from 2 435 m showed higher scores for taste intensity compared to ME wines. Furthermore, CS wines produced at 2 608 m were characterised by improved aroma intensity, aroma quality, taste purity, taste intensity, taste quality and harmony compared to the other four wines. The total score for ME wines increased with increasing altitude. Cabernet Sauvignon wine produced at 2 608 m was described as "very good", with a score of 84.1. The four other wine samples belong to the "good" level. This was in agreement with work done by King *et al.* (2014), who found that Malbec wines made from grapes originating from higher altitudes were considered more complex than those from vineyards at a lower altitude (King *et al.*, 2014). Cabernet Sauvignon wines from higher altitudes have also been found to possess clearer "red fruits" and "jam" aromas (Falcão *et al.*, 2007).

Regression analysis of terroir parameters, phenolic characteristics and antioxidant activity of red wines

LASSO is a regression analysis method that performs both variable selection and regularisation in order to enhance the prediction accuracy and interpretability of the statistical model it produces (Tibshirani, 1996). LASSO involves penalising the absolute size of the regression coefficients. The larger the penalty applied, the further the estimates are shrunk towards zero, which means the variable would be excluded from the independent variable pool. Moreover, all the independent variables could be analysed simultaneously, which is different from the traditional analysis using the step-by-step method.

Factors such as altitude and climatic conditions can have an impact on the phenolic characteristics and antioxidant activity of red wines. However, the separate contribution of each factor to the phenolic characteristics of wine should be clarified. Hence, 11 independent variables, including altitude and 10 climate parameters, as well as 21 dependent variables, comprising phenolic characteristics and antioxidant properties, were used to model the regression relationship among them by LASSO analyses. Typical trace plot coefficients as fitted by LASSO are presented in Fig. 2.

The regression coefficients between the dependent

and independent variables were determined in this study. The number of dependent variables correlating positively with the independent variable is a primary factor while the independent variables are optimised. Moreover, the regression coefficients with a higher cumulative value should be considered simultaneously (Tibshirani, 1996). Six independent variables, namely altitude, annual minimum temperature, relative humidity, rainfall, sunshine hours and wind speed, showed a positive correlation with the dependent variables based on the accumulative value of the coefficients. The highest accumulative value of the coefficients was determined for sunshine hours, which was associated with six dependent variables. Rainfall as an independent variable was correlated with only two dependent variables, although a high cumulative value of the regression coefficient was observed. A more effective regression model was obtained for altitude, because altitude was positively correlated with 10 dependent variables and showed a relatively high cumulative value of coefficients. Hence, altitude might act as a more effective independent variable and play an important role in its effect on phenolic characteristics and the antioxidant activity of red wines in the high-altitude region.
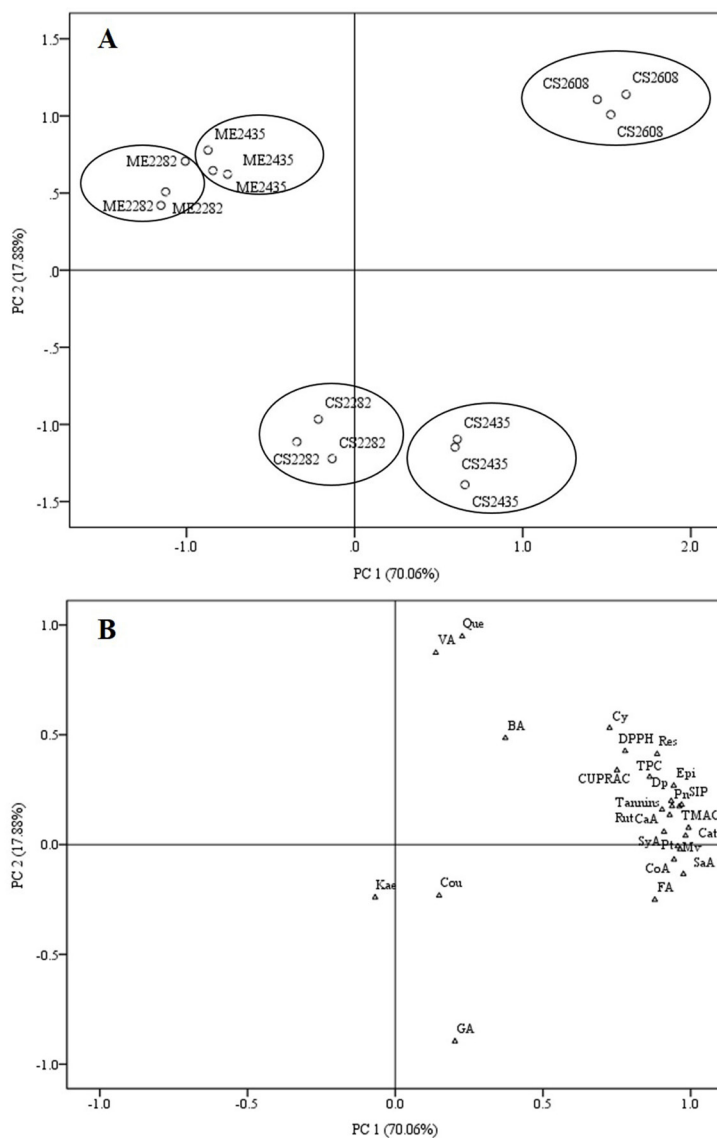

FIGURE 1

Principal component score plot (A) and correlation scatterplots (B) of the variables, with PC1 and PC2 based on the phenolic characteristics and antioxidant activity of wines from different altitudes. ME2282, CS2282, ME2435, CS2435 and CS2608 refer to Merlot wine at 2 282 m, Cabernet Sauvignon wine at 2 282 m, Merlot wine at 2 435 m, Cabernet Sauvignon wine at 2 435 m and Cabernet Sauvignon wine at 2 608 m. BA: benzoic acid; CaA: caffeic acid; Cat: (+)-catechin; CoA: *p*-coumaric acid; Cou: coumarin; CUPRAC: cupric-reducing antioxidant capacity; Cy: cyanidin-3-*O*-monoglucoside; Dp: delphindin-3-*O*-monoglucoside; DPPH: 2, 2-diphenyl-1-picrylhydrazyl free radical-scavenging activity; Epi: (−)-epicatechin; FA: ferulic acid; GA: gallic acid; Kae: kaempferol; Mv: malvidin-3-*O*-monoglucoside; Pn: peonidin-3-*O*-monoglucoside; Pt: petunidin-3-*O*-monoglucoside; Que: quercetin; Res: *trans*-resveratrol; Rut: rutin; SaA: salicylic acid; SIP: sum of individual phenolic compound; SyA: syringic acid; TMAC: total monomeric anthocyanin content; TPC: total phenolic content; VA: vanillic acid.

TABLE 8
Sensory analysis of wines from different altitudes and cultivars.

Sensory attributes		Wine				
		ME2282	CS2282	ME2435	CS2435	CS2608
Visual analysis	Clarity (5)	4.2 ± 0.2a	4.2 ± 0.1a	4.2 ± 0.2a	4.1 ± 0.1a	4.2 ± 0.3a
	Appearance (10)	6.7 ± 0.4a	6.9 ± 0.4a	7.1 ± 0.5a	7.1 ± 0.2a	7.4 ± 0.4a
Aroma analysis	Aroma purity (6)	4.0 ± 0.3a	4.1 ± 0.2a	4.2 ± 0.1a	4.0 ± 0.2a	4.1 ± 0.3a
	Aroma intensity (8)	6.0 ± 0.3b	6.2 ± 0.2b	6.0 ± 0.2b	6.2 ± 0.3b	6.6 ± 0.3a
	Aroma quality (16)	12.0 ± 0.6b	12.2 ± 0.7b	12.1 ± 0.5b	12.8 ± 0.5b	13.5 ± 0.7a
Taste analysis	Taste purity (6)	4.1 ± 0.2b	4.0 ± 0.3b	4.0 ± 0.1b	4.2 ± 0.2b	4.5 ± 0.3a
	Taste intensity (8)	6.2 ± 0.4c	6.4 ± 0.5c	6.3 ± 0.2c	6.7 ± 0.4b	7.2 ± 0.5a
	Taste prolongation (8)	6.9 ± 0.5a	7.0 ± 0.5a	7.1 ± 0.6a	7.3 ± 0.4a	7.1 ± 0.5a
	Taste quality (22)	17.1 ± 1.2c	19.0 ± 1.4b	18.5 ± 1.5b	19.1 ± 1.2b	19.6 ± 1.4a
Global evaluation	Harmony (11)	8.8 ± 0.6c	9.5 ± 0.7b	9.1 ± 0.6b	9.4 ± 0.5b	9.9 ± 0.5a
Total *	100	76.0 ± 4.8c	79.5 ± 5.2b	78.6 ± 5.4b	80.9 ± 6.2b	84.1 ± 6.1a

ME2282, CS2282, ME2435, CS2435 and CS2608 refer to Merlot wine at 2 282 m, Cabernet Sauvignon wine at 2 282 m, Merlot wine at 2 435 m, Cabernet Sauvignon wine at 2 435 m and Cabernet Sauvignon wine at 2 608 m, respectively.

* ≥ 86 = excellent; 81.0 to 85.9 = very good; 71.0 to 80.9 = good; 50.0 to 70.9 = regular; < 50 = inadequate.
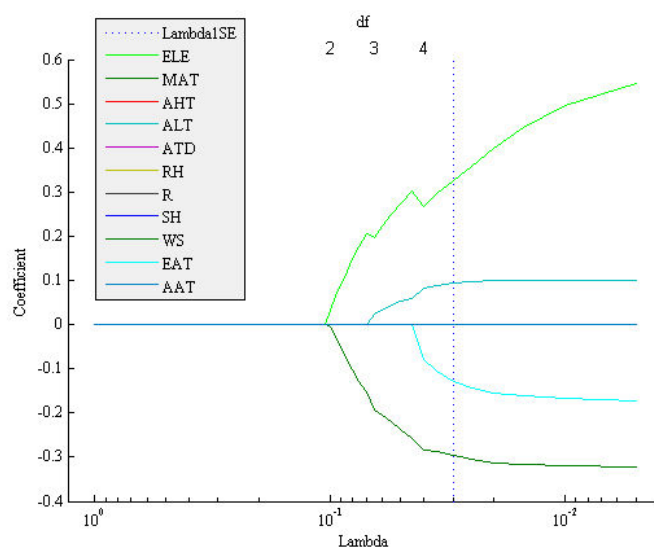

FIGURE 2
Trace plot coefficients fitted by LASSO for DPPH as a dependent variable. Independent variables were altitude (ELE), mean annual temperature (MAT), annual highest temperature (AHT), annual lowest temperature (ALT), average temperature difference (ATD), relative humidity (RH), rainfall (R), sunshine hours (SH), wind speed (WS), effective accumulative temperature (EAT) and active accumulated temperature (AAT). Each curve represents a coefficient as a function of the LASSO parameters. The intercept was not plotted. The vertical broken line represents the optimal model selected by generalised cross-validation.

CONCLUSIONS
The results of this study suggest that total phenolic, total flavonoid, and total anthocyanin content of ME and CS wines increased with altitude in high-altitude areas. Malvidin-3-O-monoglucoside was the primary monomeric anthocyanins and increased with increasing altitude. CS wines originating from higher altitude showed richer tannins. A greater antioxidant capacity was reported for ME and CS wines made from grapes originating from higher altitude vineyards. Salicylic acid, syringic acid, caffeic acid,

(+)-catechin, (−)-epicatechin, and the sum of individual phenolic compounds in wines increased with altitude. The scores of sensory evaluation for ME wines increased with higher altitude. Highest score was determined for CS wine originating from 2 608 m. PCA suggested a clear association of wines with grape cultivar and vineyard altitude. It was found that altitude followed by sunshine hours made the greatest contribution to the differences in phenolic characteristics and antioxidant activity of red wines at different sites in a high-altitude region.

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