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**EFFECT OF GROWING ZONE AND VINTAGE ON THE PREDICTION OF
EXTRACTABLE FLAVANOLS IN WINEGRAPE SEEDS BY A FT-NIR
METHOD**

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ABSTRACT

The feasibility of Fourier Transform-Near Infrared (FT-NIR) spectroscopy for predicting the extractable content of phenolic compounds directly in intact grape seeds was evaluated. NIR calibration models were based on the correlation of spectral data with the phenolic composition determined by reference chemical methods for 40 grape samples. The vintage effect was studied evidencing that the predictive accuracy improved only for spectrophotometric indices when samples of two years were simultaneously considered. The statistical parameters of calibration showed that the models developed are sufficiently robust for quantitative purposes of total flavonoids, proanthocyanidins, low molecular weight flavanols, catechin, epicatechin, procyanidin B₁ and galloylation percentage (standard prediction error=SEP%<15, predictive index=RPIQ>3.0), but could be also useful for screening of absorbance at 280 nm, total polymers, epicatechin gallate and procyanidin B₂ (SEP%<15, RPIQ=2.7-2.9). Although a calibration model is required for each geographical origin, the results suggest that FT-NIR spectroscopy is a promising analytical technique in this field.

Keywords: extractable phenols, grape seeds, red grapes, near infrared spectroscopy, chemometrics, partial least squares regression

INTRODUCTION

The most abundant phenolic compounds in grape seeds are flavanols that include monomeric catechins ((+)-catechin, (-)-epicatechin and (-)-epicatechin-3-*O*-gallate), as well as their oligomeric and polymeric procyanidins, although gallic acid is also well represented.¹⁻⁴ The qualitative and quantitative phenolic composition of the seeds greatly depends on the grape cultivar, but it is also influenced by multiple factors like environmental conditions, cultural practices and degree of grape ripeness.^{2,4-8} Seed flavanols contribute significantly to wine sensory quality because the large majority of extractable monomeric flavanols and galloylated procyanidins of the grape is located in the seeds.² In fact, the decreased content of seed monomer flavanols, particularly (-)-epicatechin-3-*O*-gallate, and the increased one of the polymeric fraction in ripe grapes are sensory perceived as low astringency and bitterness.^{1,9,10} Therefore, the flavanolic profile of the seeds is responsible for the sensations perceived in the mouth.¹

The most commonly used analytical methods for the determination of phenolic compounds in grape seeds require a preliminary step of solvent extraction.¹¹⁻¹³ Therefore, sample preparation is one of the most time consuming steps for qualitative and quantitative analysis. The use of microwave assisted extraction permits partially to reduce solvent consumption and to short extraction times.¹⁴ Nevertheless, the necessity of real-time decision-making in the oenological sector has promoted the development of rapid methods of analysis with minimal sample preparation or even on intact samples.

Near Infrared (NIR) spectroscopy is an accurate, fast, versatile, non-destructive, simple, environmental friendly and economically reasonable analytical technique that has been

widely used in the grape sector to monitor the ripening and dehydration processes, or to discriminate varieties, according to different parameters like titratable acidity, pH and the content of soluble solids, reducing sugars, moisture and organic acids.¹⁵⁻¹⁸ Regarding phenolic compounds, some studies have been published on the determination of these compounds in grape homogenates.¹⁹⁻²¹ Nowadays, the research is more focused on the direct analysis of intact grape berries, and the determination of total anthocyanins,¹⁷⁻¹⁹ extractable anthocyanins (at pH 1.0 and 3.2) and total phenols²² has been attended. Ferrer-Gallego et al. (2011) have recently determined the concentration of the main families of phenolic compounds (flavanols, anthocyanins, flavonols and phenolic acids) and their total content in grape skins and intact red grapes during ripening.²³ In intact grape seeds, only two works have assessed the possible use of NIR spectroscopy to determine phenolic compounds. In the first one, total phenol content and extractability were evaluated.²⁴ In the other one, flavanols were determined.²⁵ However, a detailed study focused on the effect of the growing zone and vintage on the robustness of the predictions for the phenolic composition has not been performed update, particularly in winegrape seeds. Other important advantage of NIR spectroscopy is the feasibility to the fast and simultaneous evaluation of several metabolites (metabolic fingerprinting) in a large number of samples.^{26,27}

The aim of this work was to develop a method based on Fourier Transform-Near Infrared (FT-NIR) spectroscopy for the direct determination of the extractable content of phenolic compounds, particularly flavanols, in intact grape seeds. To obtain a sufficiently robust predictive model, high variability calibration data set was used by sampling grapes from multiple vineyards at different ripening stages during two vintages. Furthermore, this work evaluated the vintage effect on the robustness of the

predictive model. Its adaptability to different growing zones was also checked. The study was carried out on Nebbiolo winegrapes usually used for the production of renowned red wines that are commercialized worldwide and of recognized international prestige. Therefore, this work also aimed to contribute to assess the phenolic composition of Nebbiolo seeds during ripening, while shorting the time involved in the chemical analysis methodologies commonly used for determining the extractable content of phenolic compounds in grape seeds.

MATERIALS AND METHODS

Grape Samples. A total of 40 grape samples of the Nebbiolo red cultivar (*Vitis vinifera* L.) were used for the development of the FT-NIR method. The bunches (n = 25) were randomly harvested from the vines selected in 2010 and 2011. In the two years, the samples were collected at different ripening stages in ten commercial vineyards with a total range of soluble solids content comprised between 21.6 and 25.5 °Brix. These vineyards are located in Valtellina (Sondrio province, Lombardy, Northern Italy) and confined over nearly 50 km, with elevations ranging from 300 up to 700 m a.s.l., along the southern slope of a typical valley of an alpine glacial. This is pedologically divided into four landscape units (alluvial fans, terraces, mountainside glacial tills and valley scarp), but the vineyards are mainly located in the valley scarp landscape.²⁸ The geographical distribution of the vineyards selected in the Valtellina valley is shown in Figure S1. In 2011, other six samples were harvested at different ripening stages (total range of soluble solids content from 19.5 to 23.9 °Brix) from vineyards located in Piedmont (Cuneo province, North-West Italy) and Trentino (Trento province, North-

East Italy) with altitudes of 239 and 262 m a.s.l., respectively, and were used to evaluate the production zone effect. The location and geographical coordinates of the different vineyards studied are shown in Table S1. On the other hand, the climatic conditions of each vintage in terms of temperature, relative humidity and precipitations are represented in Figure S2.

The analyses were carried out separately on the samples collected at each date, vineyard and year. Once in the laboratory, a subsample of approximately 0.5 kg of grapes (ca. 350-400 berries) was randomly selected by picking berries from different positions in the cluster. For each subsample, two sets of 30 berries were randomly selected. The first set was subdivided into three replicates of 10 grape berries that were weighed. The seeds were carefully separated from the pulp, cleaned with adsorbent paper, and weighed before determining extractable phenolic compounds by spectrophotometric and chromatographic methods, which were taken as the reference (n=3). In the second set, the seeds were also separated and cleaned before FT-NIR analysis. In this last case, one seed per berry was used to cover a wider variation range with the same number of seeds. The FT-NIR spectrum was individually acquired in these 30 intact grape seeds (n=30). Finally, the remaining berries of each initial subsample, distributed into three replicates, were used for determining the technological ripeness parameters in the grape must obtained by manual crushing and centrifugation.

Chemical Analysis. Solvents of HPLC-gradient grade and all other chemicals of analytical-reagent grade were purchased from Sigma (Milan, Italy). The solutions were prepared in deionized water produced by a Purelab Classic system (Elga Labwater, Marlow, United Kingdom). Among phenol standards, (+)-catechin (C), (-)-epicatechin

(EC) and (-)-epicatechin gallate (ECG) were obtained from Sigma, and cyanidin chloride, procyanidins B₁ and B₂ were purchased from Extrasynthèse (Genay, France). Phloroglucinol was supplied by Aldrich (Steinheim, Germany).

Technological Ripeness Parameters. Total soluble solids concentration (°Brix, as SSC) was measured with an Atago 0–32 °Brix temperature compensating refractometer (Atago Corporation, Tokyo, Japan), and pH was determined by potentiometry using a Crison electrode (Carpi, Italy). Titratable acidity (TA), expressed as g L⁻¹ tartaric acid, was estimated using the International Organization of Vine and Wine (O.I.V.) method.²⁹ Organic acids (malic acid and tartaric acid) were quantified (as g L⁻¹) according to the methodology proposed by Giordano et al. (2009).³⁰

Seed Phenol Extraction and Determination. In the reference method, the berry seeds, after immersion in 50 mL of a hydroalcoholic buffer at pH 3.2 containing 5 g L⁻¹ tartaric acid, 2 g L⁻¹ Na₂S₂O₅ and 12% (v/v) ethanol, were placed in a controlled temperature room at 25 °C for one week.^{31,32} The extracts were filtered through a 0.20 µm filter, bottled and stored at 4 °C until their analysis. Spectrophotometric methods were used to measure seed absorbance at 280 nm ($A_{s,280}$),³³ and to determine the extractable content of seed total flavonoids (TF_s, mg (+)-catechin kg⁻¹ grape or g⁻¹ seed), seed proanthocyanidins (PRO_s, mg cyanidin chloride kg⁻¹ grape or g⁻¹ seed) and seed flavanols reactive to vanillin (FRV_s, mg (+)-catechin kg⁻¹ grape or g⁻¹ seed).^{31,34} Proanthocyanidins were determined after acid hydrolysis with warming (Bate-Smith reaction) using a ferrous salt (FeSO₄) as catalyst. An UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) was used.

Individual flavanols were determined by reversed-phase liquid chromatography before and after acid-catalyzed degradation of polymeric proanthocyanidins in the presence of phloroglucinol. Phloroglucinolysis was carried out according to the method proposed by Kennedy and Jones (2001),³⁵ although slightly modified as follows. One milliliter of the hydroalcoholic extract was evaporated to dryness under reduced pressure at 35 °C. The residue was redissolved in 0.5 mL of phloroglucinol reagent consisting of 50 g L⁻¹ phloroglucinol and 10 g L⁻¹ ascorbic acid in methanol containing 0.1 M hydrochloric acid. After reacting for 20 min at 50 °C, the reaction was stopped by adding 0.5 mL of 200 mM aqueous sodium acetate. The final extracts (taken before and after phloroglucinolysis) were filtered through 0.20 μm PTFE filters (VWR International, Radnor, PA, US) into LC vials and immediately injected into a HPLC-DAD system. This assay provides information on the extractable content of total polymer flavanols (TP), the mean degree of polymerization (mDP) and the percentage of galloylation (G%).

An Agilent 1260 Infinity HPLC system (Milford, MA, USA) equipped with a diode array detector (DAD) was used to determine individual flavanols. The chromatographic separation was carried out at 25 °C on a LiChroCART analytical column (250 mm × 4.0 mm i.d.) purchased from Merck (Darmstadt, Germany), which is packed with LiChrospher 100 RP-18 (5 μm) particles supplied by Alltech (Deerfield, IL, USA). The injection volume was 20 μL. The mobile phases consisted of 1% aqueous acetic acid (mobile phase A) and methanol (mobile phase B). They were filtered through a 0.20 μm filter. A linear gradient, at a flow rate of 0.8 mL min⁻¹, was used for the separation of flavanols in 55 min, starting at 5% B for 10 min, increasing to 20% B in 20 min and to 40% B in 25 min. The column was then washed with 90% B for 10 min and equilibrated

with 5% B for 5 min prior to each analysis.³⁵ The UV-VIS spectra were acquired from 230 to 400 nm, and the detection wavelength was set at 280 nm. The identification of monomer and dimer flavanols was achieved by comparing their absorption spectra and retention times with those of pure standards. The quantification (mg kg^{-1} grape or mg g^{-1} seed) was carried out by the external standard method. The phloroglucinol adducts were identified on the basis of their retention times and quantified as equivalents of their respective free flavanol (external standard method), assuming the same molar absorptivity between each free flavanol and its corresponding phloroglucinol adduct.³⁶ The mDP value was calculated as the molar ratio of the sum of all flavanol units produced by phloroglucinolysis (phloroglucinol adducts plus monomers) to the sum of monomer flavanols. The G% value was calculated as the ratio of the sum of galloylated flavanols to the sum of all flavanols. All analyses were performed in duplicate.

Spectral and Chemometric Analysis. Intact berry seeds were individually scanned using a NIRFlex N500 spectrophotometer equipped with Solids Transmittance module (Buchi, Flawil, Switzerland). The FT-NIR spectra were collected in transmittance mode within the $4000\text{-}12000\text{ cm}^{-1}$ wavenumber range at 4 cm^{-1} resolution using the NIR-Operator software (Buchi, Flawil, Switzerland). For each seed, a total of 64 scans were acquired on the lateral side and were averaged for each transmittance spectrum.²⁴ All NIR analyses were carried out at room temperature ($20 \pm 1^\circ\text{C}$).

All spectra were randomly subdivided into the calibration set (about 2/3) and the validation set (about 1/3), the two sets being associated with comparable ranges of phenolic compounds. Principal component analysis (PCA) was performed before regression to provide information on the latent structure of the spectral data. This

permits to evidence possible grouping samples and to detect the presence of outliers. The FT-NIR spectra were pre-treated, and a spectral region was selected to minimize the standard error of prediction (SEP).³⁷ Table 1 shows the spectral pre-treatments applied, the number of principal components (PCs), and the wavenumbers selected using the NIRCAL 5.2 software (Buchi, Flawil, Switzerland). Calibration models based on the phenolic composition determined by the reference method and the NIR spectra were performed by partial least squares (PLS) regression using the NIRCAL 5.2 software.

PCA was an effective tool in addressing the classification of samples according to the growing zone, verifying whether unknown Piedmont and Trentino samples do not belong to the spectral space created by the Valtellina samples involved in the development of the regression equations. Other statistical analyses were performed using the SPSS software package version 19.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to establish significant differences in the chemical parameters or loadings of principal components among growing zones.

RESULTS AND DISCUSSION

Chemical Analysis. Nebbiolo winegrapes from Valtellina were sampled at different dates to consider the natural variability in the ripening stage that influences both the technological ripeness parameters and the phenolic composition. Furthermore, the samples were collected in ten vineyards to consider the agroclimatic variability (Table 1S). In this way, a wider compositional range of the grape was covered. Figure 1 shows

the variation range of the technological ripeness parameters for Nebbiolo winegrapes from Valtellina in the two vintages studied (2010 and 2011). In 2010, the results obtained for all of the technological ripeness parameters evidenced higher variability than in 2011, as can be observed in the variation range and in the interquartile amplitude. The values represented in Figure 1 vary within the range expected for Nebbiolo winegrapes from Valtellina,²⁸ and practically cover the variability during ripening in Piedmont growing areas.³⁸

Because of little scientific contributions currently available regarding the phenolic composition of Nebbiolo seeds, a comparison was established with other winegrape varieties with the aim of contributing to a better characterization of the Nebbiolo cultivar. At the last harvest date, the extractable content of total flavonoids ranged from 70.9 to 103.8 mg g⁻¹ seed in 2010 (2697-4047 mg kg⁻¹ grape), and from 32.4 to 54.7 mg g⁻¹ seed in 2011 (1240-1802 mg kg⁻¹ grape). The vintage effect was also observed on the content of total flavanols in Graciano seeds, the variation range being 25.3-40.9 mg g⁻¹ seed during 2005-2008.¹ The Nebbiolo cultivar seems to evidence higher concentrations of total flavonoids in the seeds than the Graciano cultivar. Other works also reported lower concentrations of total flavanols for Cencibel, Cabernet Sauvignon, Merlot and Shiraz seeds (330-870 mg kg⁻¹ grape),⁴ whereas values of total flavonoids of ca. 50 mg g⁻¹ seed were found in Vranec and Merlot seeds.³⁹

The extractable concentration of high molecular weight proanthocyanidins, determined by spectrophotometry and expressed as PRO_s, for Nebbiolo winegrapes at harvest varied between 20.5 and 33.7 mg g⁻¹ seed in 2010 (837-1351 mg kg⁻¹ grape), and between 12.5 and 29.3 mg g⁻¹ seed in 2011 (492-999 mg kg⁻¹ grape). These are

comprised between the values reported for Carménère, Merlot, Cabernet Franc and Cabernet Sauvignon seeds (6.6-16.2 mg g⁻¹ seed)⁷ and the found for Malbec seeds (99.2-125.2 mg g⁻¹ seed).⁵ On the other hand, the extractable content of simple monomer flavanols together with low molecular weight oligomer flavanols, determined by spectrophotometry and expressed as FRV_s,⁴⁰ in Nebbiolo seeds at the harvest date ranged from 8.8 to 13.4 mg g⁻¹ seed in 2010 (314-610 mg kg⁻¹ grape), and from 10.3 to 29.7 mg g⁻¹ seed in 2011 (405-828 mg kg⁻¹ grape). These values agreed with those reported for Vranec and Merlot seeds (16.7 and 18.4 mg g⁻¹ seed, respectively),³⁹ and for Malbec ones (10.8-11.9 mg g⁻¹ seed).⁵

Regarding monomer and dimer flavanols, determined by HPLC, the extractable concentration in the seeds of Nebbiolo winegrapes at harvest ranged from 1.50 to 3.15, from 1.04 to 1.57, from 0.04 to 0.09, from 0.43 to 0.66 and from 0.36 to 0.55 mg g⁻¹ seed in 2010 for (+)-catechin (C), (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), procyanidins B₁ and B₂, respectively (60.6-126.6, 42.1-63.3, 1.6-3.6, 17.5-29.9 and 14.6-23.7 mg kg⁻¹ grape, respectively), and from 1.52 to 2.97, from 0.92 to 1.59, from 0.03 to 0.05, from 0.35 to 0.73 and from 0.37 to 0.76 mg g⁻¹ seed in 2011 for C, EC, ECG, procyanidins B₁ and B₂, respectively (59.7-109.1, 28.9-57.8, 1.1-1.6, 14.1-21.0 and 14.5-23.5 mg kg⁻¹ grape, respectively). It supposes a variation range for total monomer flavanols (TM) of 2.60-4.81 mg g⁻¹ seed in 2010 and 2.47-4.33 mg g⁻¹ seed in 2011 (104.6-193.5 and 97.1-163.0 mg kg⁻¹ grape, respectively). Nebbiolo seeds accounted for an extractable content of total polymer flavanols (TP) in the range of 6.81-10.26 mg g⁻¹ seed in 2010 and 5.93-11.79 mg g⁻¹ seed in 2011 (291.1-449.0 and 223.3-416.3 mg kg⁻¹ grape, respectively). The extractable content of individual monomer flavanols in Nebbiolo seeds agreed with that reported for other autochthonous

and international red winegrape varieties grown in Trentino.² At harvest, the most abundant monomer flavanol in Nebbiolo seeds was C (58-73%), followed by EC (26-41%). Furthermore, the extractable content of polymeric proanthocyanidins agreed with the values published for Nebbiolo seeds at the last ripening stages, EC-phloroglucinol being the more representative adduct in the extension units.⁴¹ On the other hand, the values of the mean degree of polymerization (mDP) and the galloylation percentage (G%) (4.0-7.2, 5.3-12.1%) were slightly lower than others published for Nebbiolo seeds at the last ripening stages (9.5-10.7, 13.1-14.7%).⁴¹ This can be supported by a poorer extraction of large proanthocyanidins in the wine-like solution used, and/or by possible depolymerization as a consequence of the acidity of the hydroalcoholic solution buffered at pH 3.2.²

Figures 2 and 3 show the variation range for the measurement of $A_{s,280}$ and for the extractable content of phenolic compounds in the seeds from Nebbiolo winegrapes collected, in Valtellina, at different stages of maturity considering each year separately. The median was higher in 2010 for $A_{s,280}$, TF_s , PRO_s , ECG and procyanidin B_1 , but lower for FRV_s , C, EC, TM, procyanidin B_2 and TP. The lowest variation range and interquartile amplitude were associated with 2010, excepting for TF_s . The vintage effect was particularly evident on the extractable content of total flavonoids because of the completely different variation range for the two years studied. This variability in the phenolic composition of Nebbiolo seeds was considered suitable for developing NIR calibrations. In fact, the variation range covers the most typical values reported for the extractable content of total flavonoids, polymeric proanthocyanidins and low molecular weight flavanols in the seeds of Nebbiolo winegrapes, at harvest, from several production zones located in Piedmont.^{41,42}

FT-NIR Analysis. Tables 2 and 3 show the mean, minimum and maximum values in both the calibration and validation sets, respectively, for the measurement of $A_{s,280}$ and the extractable content of phenolic compounds in the seeds determined by the reference method (extraction combined with UV-visible spectrophotometry or liquid chromatography). The performance of PLS calibration models was assessed from the correlation coefficient of calibration (R_c) and the standard error of calibration (SEC). The standard error of calibration was also standardized (SEC%) by rating its value to the mean of the calibration population, and is related to the mean error of the model. A good calibration model should have high R_c and low SEC and SEC%. With exception of $A_{s,280}$ per weight of seed, the best statistical parameters of calibration for the spectrophotometric indices were obtained when the calibration sets corresponding to the two years studied were simultaneously considered ($R_c > 0.80$, $SEC\% < 16$) (Table 2). Regarding flavanolic compounds determined by HPLC, the best statistical parameters of calibration for individual monomer flavanols, total monomers and galloylation percentage were associated with 2011 vintage, whereas those for dimer flavanols, mean degree of polymerization and total polymeric proanthocyanidins were obtained in 2010 vintage ($R_c \geq 0.52$, $SEC\% \leq 19$) (Table 2). The statistical parameters evidenced that PLS calibration models could be used with quantification purposes for TF_s , FRV_s , PRO_s , individual flavanols and G%. In fact, very good calibration models between the FT-NIR spectra and the content of phenolic compounds determined by the reference method were obtained in 2010 for TF_s per weight of grape and procyanidin B₁ per weight of seed, in 2011 for FRV_s and ECG per weight of seed, and in 2010 and 2011 together for FRV_s per weight of grape ($R_c > 0.95$, $SEC\% < 9$). On the other hand, satisfactory calibration models were found in 2010 for TF_s per weight of seed and

procyanidin B₂ per weight of grape, in 2011 for TF_s, FRV_s and EC per weight of grape, C and procyanidin B₁ per weight of seed and G%, and in 2010 and 2011 together for PRO_s per weight of grape, TF_s per weight of grape or seed, FRV_s and ECG per weight of seed and G% ($R_c = 0.86-0.95$, $SEC\% \leq 15$). Because of the goodness of the statistical parameters for the Nebbiolo cultivar, the usefulness of FT-NIR spectroscopy to predict the flavanolic composition of the seeds for different red winegrape cultivars was also investigated. However, not good enough values of R_c were obtained, which coincided with excessively high SEC% values (data not shown). Therefore, the grape variety strongly affects the usefulness of PLS calibration models.

An external validation was performed to assess the robustness of PLS calibration models using a sample set that did not belong to the calibration set. The calibration equations obtained were applied to the validation set, and the chemical parameters determined in the seeds by the reference method were compared with those predicted by the NIR calibration (Table 3). The predictive accuracy of PLS calibration models was evaluated from the correlation coefficient of validation (R_v) and SEP. The goodness of the prediction ability requires high R_v and low SEP. Bellon-Maurel et al. (2010) referred to the increase in the SEP value when the measurement range – or the mean of this range – increases.⁴³ Therefore, other three statistical parameters were used to standardize the predictive accuracy of each calibration model removing any variation range effect. The coefficient of variation (SEP%) was calculated as the ratio of the SEP value to the mean of the validation population. The residual predictive deviation (RPD) was defined as the ratio between the standard deviation (SD) of the validation set and the SEP value. RPD is the most commonly used statistical index to account for the model reliability. However, a new index (RPIQ), based on quartiles, better represents

the population spread, regardless of the distribution.⁴³ This was calculated as the ratio of the interquartile amplitude of the validation population to the SEP value.

The SEP% values lower than 20 are considered acceptable for most analytical purposes,²⁰ which evidences the suitability of the NIR calibration models developed to predict the phenol content in intact berry seeds. The differences found between the reference values and those predicted by NIR calibration models were smaller ($R_v \geq 0.85$, $SEP\% < 15$) in 2010 for TF_s , PRO_s and procyanidin B_2 per weight of grape, in 2011 for TF_s and EC per weight of grape, C per weight of seed, G% and FRV_s per weight of grape or seed, and in 2010 and 2011 together for PRO_s per weight of grape, ECG per weight of seed, as well as TF_s and FRV_s per weight of grape or seed (Table 3). A better standardization of the SEP value is provided by the RPD and RPIQ indices. If the SEP value is small compared to the population spread of a certain chemical parameter, a relatively high index is obtained. Therefore, the higher the RPD value the greater the predictive accuracy. Chang et al. (2001) established that RPD values higher than 2.0 are very satisfactory for prediction purposes, whereas values ranging between 1.4 and 2.0 are indicative of fair models.⁴⁴ This would permit to account the NIR calibration models developed, using simultaneously the data sets corresponding to the two years studied, for the quantification of spectrophotometric indices related to total flavonoids per weight of grape or seed, proanthocyanidins and low molecular weight flavanols per weight of grape, as well as for screening of $A_{s,280}$ and (-)-epicatechin gallate per weight of grape or seed and galloylation percentage (Table 3). On the other hand, the NIR calibration models were sufficiently reliable for quantification or screening purposes only if constructed from the data set corresponding to 2010 vintage for the determination of total polymer flavanols per weight of grape and procyanidin B_2

per weight of grape or seed, or those of 2011 vintage for the determination of (-)-epicatechin per weight of grape, and (+)-catechin and procyanidin B₁ per weight of seed (Table 3). However, according to standards proposed by other authors (RPD = 2.4-4.9),⁴⁵ the NIR calibration models developed could be fair and recommended only for screening purposes of the extractable content of total flavonoids in both 2010 and 2011 vintages together, or of the extractable content of (+)-catechin per weight of seed and galloylation percentage in 2011 vintage. No statistical basis was used to determine these thresholds, and some researchers have begun to criticize this statistical index.⁴⁶ As above mentioned, the use of the RPIQ index provides a better evaluation of the predictive ability of NIR calibration models. Using simultaneously the data of the two years studied, the RPIQ values indicated that the predictive accuracy was very good for total flavonoids per weight of grape or seed (RPIQ \geq 5.0), satisfactory for the determination of spectrophotometric indices related to proanthocyanidins and monomer together with oligomer flavanols per weight of grape (RPIQ = 3.0-5.0), and unreliable for quantitative purposes of A_{s,280} per weight of grape and ECG per weight of grape or seed but acceptable for screening (RPIQ = 2.4-3.0). Furthermore, satisfactory predictive accuracy for (-)-epicatechin per weight of grape, procyanidin B₁ per weight of seed and galloylation percentage, and good robustness for the quantitative prediction of (+)-catechin per weight of seed were only achieved in 2011 vintage. Instead, the only use of the data set corresponding to 2010 vintage enabled the screening of procyanidin B₂ per weight of grape and total polymer flavanols per weight of seed.

The evaluation of all statistics permitted to verify that the most reliable PLS calibration models for the prediction of the spectrophotometric parameters were obtained when the sample sets of the two different vintages were considered, and the reference values were

expressed as content or absorbance per weight of grape. Nevertheless, the equations corresponding to $A_{s,280}$ could be useful only for screening purposes in the seeds. Regarding the flavanolic composition, the most robust calibration models corresponded to the individual use of the sample set of 2011 vintage.

The statistical parameters obtained for the prediction of the content of total flavonoids in the seeds by FT-NIR spectroscopy were compared with the reported for total flavanols by Ferrer-Gallego et al. (2010) for samples harvested in two vineyards located in the same growing zone (SEP = 4.9 mg g⁻¹ seed).²⁵ Although a worse value of SEP was found (8.8 mg g⁻¹ seed), the standard deviation of the sample set was also 1.7 times larger. Therefore, comparable predictive accuracy is expected. Also the extractable content of (+)-catechin, (-)-epicatechin, procyanidins B₁ and B₂ and total monomer flavanols was compared, and lower values of SEP were obtained in the present work, excepting for procyanidin B₁ with slightly higher values than the reported by Ferrer Gallego et al. (2010) as function of vintage (0.05-0.09 instead of 0.06 mg g⁻¹ seed).²⁵ Regarding the measurement of $A_{s,280}$ per weight of seed, the statistics obtained were quite similar to the previously provided by Rolle et al. (2012) ($R_v = 0.65$, SEP% = 7.8)²⁴ in spite of the fact the samples were collected in only one vineyard. The main advantage of the NIR calibration models now developed is their applicability to multiple vineyards characterized by very different agroclimatic conditions, despite being located in the same geographical area.

The necessity of developing annual calibration models for the prediction of individual monomer and dimer flavanols, as well as of total polymer flavanols and galloylation percentage, limits the practical applicability of FT-NIR spectroscopy. Therefore, the

next step was to study the growing zone effect by means of checking the predictive ability of NIR calibration models only for the spectrophotometric indices, closely related to the phenolic composition of the seeds, in the Nebbiolo cultivar from six vineyards located in other growing zones very environmentally different to Valtellina, such as Piedmont and Trentino (Table S1). The calibration equations developed for Valtellina samples were applied to Piedmont and Trentino ones, and the values predicted for $A_{s,280}$ and the extractable content of TF_s , FRV_s and PRO_s were compared with the reference data. Although the reference values for Nebbiolo seeds from Piedmont and Trentino agreed with the reported for Valtellina, with very few exceptions as can be seen in Table 4, the differences found between these values and the predicted by the NIR technique ranged from 21.6% to 76.2%, these being significant for most determinations ($p < 0.01$). In an attempt to verify if these differences were due to variations in the NIR spectra, the samples from the three growing zones were classified applying PCA to the NIR spectral data of the intact seeds. Table 5 shows the loadings of the four principal components for the samples. The two first principal components (91% overall explained variance) permitted satisfactory separation among the three zones, as demonstrated by the significant differences among loadings ($p < 0.01$). At this moment, the NIR calibration models developed only provide good predictive accuracy of the chemical parameters, usually used in cellars to determine the phenolic ripeness of the seeds, if vineyards belonging to the same production zone are sampled.

FT-NIR spectroscopy is a fast and reliable technique for predicting the extractable content of phenolic compounds in Nebbiolo seeds, particularly from grapes harvested in different vineyards located in the same growing zone. Most of chromatographic parameters, like individual monomer and dimer flavanols, total polymer flavanols and

galloylation percentage, can be only satisfactorily predicted by using NIR calibration models developed for each vintage. On the other hand, the robustness of the NIR calibration models to predict spectrophotometric indices, closely related to the phenolic composition of the seeds, increased using simultaneously the sample sets of two years, if compared with that corresponding to each individual year. This work permitted to ensure the suitability of the FT-NIR spectra on intact berry seeds as a routine analytical tool for optimizing harvest decisions, selective processing of grapes, as well as assessing and managing the phenols extraction during the winemaking process, depending on the flavanolic characteristics of the seeds. This aspect is particularly important for winegrape cultivars with a high richness in seed flavanols, as observed for the Nebbiolo cultivar if compared with other winegrape varieties. Because the grape variety, vintage and zone effect were limiting factors on the transferability of the predictive models, further works are in progress covering higher number of years, growing zones and winegrape varieties to extend the applicability of the method while improving the robustness and accuracy of the calibration models. The FT-NIR method proposed represents a good alternative to the chemical analyses of the seeds most commonly required in cellars.

Abbreviations used: $A_{s,280}$, seed absorbance at 280 nm; TF_s , seed total flavonoids; PRO_s , seed proanthocyanidins; FRV_s , seed flavanols reactive to vanillin; C, (+)-catechin; EC, (-)-epicatechin; ECG, (-)-epicatechin gallate; TM, total monomer flavanols; B_1 and B_2 , procyanidins B_1 and B_2 ; TP, total polymer flavanols; mDP, mean degree of polymerization; G%, galloylation percentage.

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Supporting Information

Location and geographical coordinates of the different vineyard zones studied, and climatic conditions of 2010 and 2011 vintages.

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FIGURE CAPTIONS

Figure 1. Technological ripeness parameters for Nebbiolo winegrapes from ten vineyards at different ripening stages in 2010 and 2011: total soluble solids concentration (Fig. 1_a), pH (Fig. 1_b), titratable acidity, malic acid concentration and tartaric acid concentration (Fig. 1_c).

Figure 2. $A_{s,280}$ (Fig. 2_{a,b}) and extractable content of TF_s, PRO_s and FRV_s (Fig. 2_{c,d}), per weight of grape (Fig. 2_{a,c}) and seed (Fig. 2_{b,d}), for Nebbiolo seeds from ten vineyards at different grape ripening stages in 2010 and 2011.

Figure 3. Extractable content of C, EC and ECG (Fig. 3_{a,b}), procyanidin B₁ and B₂ (Fig. 3_{c,d}), and TM and TP (Fig. 3_{e,f}), per weight of grape (Fig. 3_{a,c,e}) and seed (Fig. 3_{b,d,f}), for Nebbiolo seeds from ten vineyards at different grape ripening stages in 2010 and 2011.

Table 1. Spectral Pretreatments, Number of Principal Components (PCs) and Wavenumbers Selected in the Development of NIR Calibration Models for Absorbance at 280 nm and Extractable Content of Phenolic Compounds in Intact Nebbiolo Seeds Using Different Number of Vintages

| Chemical parameter | Spectral pretreatments ^a | PCs | Wavenumbers (cm ⁻¹) |
|--|-------------------------------------|-----|---------------------------------|
| <i>2010 vintage</i> | | | |
| A _{s,280} kg ⁻¹ grape | mf | 6 | 8500-11520 |
| TF _s (mg kg ⁻¹ grape) | mf,dg1 | 7 | 6000-7200 |
| PRO _s (mg kg ⁻¹ grape) | SNV,dg1 | 5 | 6000-6800 |
| FRV _s (mg kg ⁻¹ grape) | SNV,db1 | 5 | 6000-6800 |
| C (mg kg ⁻¹ grape) | SNV,db1 | 2 | 7400-10600 |
| EC (mg kg ⁻¹ grape) | SNV,db1 | 2 | 7400-10600 |
| ECG (mg kg ⁻¹ grape) | mf | 4 | 6000-10600 |
| TM (mg kg ⁻¹ grape) | SNV,db1 | 1 | 7400-10600 |
| B ₁ (mg kg ⁻¹ grape) | sa3,ncl,db1 | 5 | 7400-11520 |
| B ₂ (mg kg ⁻¹ grape) | db1,ncl | 6 | 6000-6800 |
| TP (mg kg ⁻¹ grape) | db1,ncl | 3 | 8500-11520 |
| mDP | SNV | 2 | 7400-11520 |
| G% | db1,ncl | 4 | 6000-6600, 7800-11520 |
| A _{s,280} g ⁻¹ seed | mf,dg2 | 2 | 8500-11520 |
| TF _s (mg g ⁻¹ seed) | mf,dg2 | 2 | 6000-11520 |
| PRO _s (mg g ⁻¹ seed) | dg1,mf | 2 | 7400-11520 |
| FRV _s (mg g ⁻¹ seed) | mf,dg1 | 5 | 8500-11520 |
| C (mg g ⁻¹ seed) | sa3,ncl,db1 | 2 | 6000-10600 |
| EC (mg g ⁻¹ seed) | mf | 2 | 6000-10600 |
| ECG (mg g ⁻¹ seed) | mf | 3 | 6000-10600 |
| TM (mg g ⁻¹ seed) | dg1,nle | 2 | 8500-11520 |
| B ₁ (mg g ⁻¹ seed) | sa3,ncl,db1 | 6 | 8500-10600 |
| B ₂ (mg g ⁻¹ seed) | mf | 4 | 6000-10600 |
| TP (mg g ⁻¹ seed) | dg1,nle | 2 | 6000-11520 |
| <i>2011 vintage</i> | | | |
| A _{s,280} kg ⁻¹ grape | ncl | 2 | 6000-6600, 7800-11520 |
| TF _s (mg kg ⁻¹ grape) | dg1,SNV | 7 | 8500-10600 |
| PRO _s (mg kg ⁻¹ grape) | ncl,db1 | 4 | 6000-10000 |
| FRV _s (mg kg ⁻¹ grape) | SNV | 5 | 6000-6800 |
| C (mg kg ⁻¹ grape) | mf | 4 | 6000-10600 |
| EC (mg kg ⁻¹ grape) | dg2,SNV | 4 | 7400-11520 |
| ECG (mg kg ⁻¹ grape) | mf,dg2 | 3 | 8500-11520 |

| | | | |
|--|-------------|----|-----------------------|
| TM (mg kg ⁻¹ grape) | ncl,db1 | 6 | 8500-11520 |
| B ₁ (mg kg ⁻¹ grape) | SNV,dg1 | 2 | 6000-6800 |
| B ₂ (mg kg ⁻¹ grape) | SNV | 2 | 6000-9800 |
| TP (mg kg ⁻¹ grape) | ncl,db1 | 4 | 6000-6800 |
| mDP | db1,ncl | 2 | 7400-11520 |
| G% | dg1,mf | 6 | 7400-11520 |
| A _{s,280} g ⁻¹ seed | mf | 3 | 7400-11520 |
| TF _s (mg g ⁻¹ seed) | dg2,SNV | 6 | 8500-10600 |
| PRO _s (mg g ⁻¹ seed) | SNV | 2 | 6000-7200 |
| FRV _s (mg g ⁻¹ seed) | SNV,dg2 | 6 | 6000-11520 |
| C (mg g ⁻¹ seed) | mf,dg2 | 4 | 6000-11520 |
| EC (mg g ⁻¹ seed) | mf,dg2 | 2 | 8500-11520 |
| ECG (mg g ⁻¹ seed) | n01,db1 | 8 | 8500-11520 |
| TM (mg g ⁻¹ seed) | ncl | 6 | 6000-10600 |
| B ₁ (mg g ⁻¹ seed) | SNV,dg2 | 3 | 6000-6600, 7800-11520 |
| B ₂ (mg g ⁻¹ seed) | mf | 2 | 8500-10600 |
| TP (mg g ⁻¹ seed) | mf,dg2 | 2 | 6000-6800 |
| <i>2010 and 2011 vintages</i> | | | |
| A _{s,280} kg ⁻¹ grape | SNV,dg2 | 5 | 6000-7100, 7400-10000 |
| TF _s (mg kg ⁻¹ grape) | db1,ncl | 6 | 6000-9200 |
| PRO _s (mg kg ⁻¹ grape) | dg2,SNV | 6 | 6000-7100, 7400-9600 |
| FRV _s (mg kg ⁻¹ grape) | dg1,mf | 10 | 6000-10000 |
| C (mg kg ⁻¹ grape) | mf,db1,nle | 6 | 7400-10600 |
| EC (mg kg ⁻¹ grape) | sa3,ncl,db1 | 2 | 6000-11520 |
| ECG (mg kg ⁻¹ grape) | dg2,SNV | 4 | 6000-10000 |
| TM (mg kg ⁻¹ grape) | db1,SNV | 4 | 8500-11520 |
| B ₁ (mg kg ⁻¹ grape) | dg2,SNV | 2 | 6000-9200 |
| B ₂ (mg kg ⁻¹ grape) | SNV,dg2 | 3 | 8500-10600 |
| TP (mg kg ⁻¹ grape) | db1,SNV | 6 | 6000-7200 |
| mDP | SNV,db1 | 2 | 6000-7200 |
| G% | db1,SNV | 8 | 7400-11520 |
| A _{s,280} g ⁻¹ seed | ncl | 4 | 6000-11520 |
| TF _s (mg g ⁻¹ seed) | dg1,SNV | 8 | 6000-8800 |
| PRO _s (mg g ⁻¹ seed) | dg1,nle | 7 | 7400-11520 |
| FRV _s (mg g ⁻¹ seed) | dg2,SNV | 7 | 6000-9600 |
| C (mg g ⁻¹ seed) | dg1,mf | 3 | 8500-10600 |
| EC (mg g ⁻¹ seed) | dg2,SNV | 2 | 6000-7144, 7404-11520 |
| ECG (mg g ⁻¹ seed) | SNV,db1 | 6 | 8500-11520 |

| | | | |
|--|---------|---|-----------------------|
| TM (mg g ⁻¹ seed) | dg2,SNV | 3 | 6000-10600 |
| B ₁ (mg g ⁻¹ seed) | mf | 3 | 6000-9800 |
| B ₂ (mg g ⁻¹ seed) | dg2,SNV | 2 | 6000-6600, 7800-11520 |
| TP (mg g ⁻¹ seed) | dg1,SNV | 3 | 8500-11520 |

^amf = full multiplicative scatter correction, dg1 = first derivative Savitzky-Golay (9 points), SNV = standard normal variate, db1 = first derivative BCAP, sa3 = smooth average (3 points), ncl = normalization by closure, dg2 = second derivative Savitzky-Golay (9 points), nle = normalization to length unit, n01 = normalization between 0 and 1.

Table 2. Statistical Parameters of NIR Calibration Models Developed for Absorbance at 280 nm and Extractable Content of Phenolic Compounds in Intact Nebbiolo Seeds Using Different Number of Vintages

| Chemical parameter | Mean | Minimum | Maximum | R _c ^c | SEC ^d | SEC% ^e |
|--|-------|---------|---------|-----------------------------|------------------|-------------------|
| <i>2010 vintage^a</i> | | | | | | |
| A _{s,280} kg ⁻¹ grape | 17.8 | 12.1 | 23.0 | 0.77 | 2.0 | 11.5 |
| TF _s (mg kg ⁻¹ grape) | 3304 | 2776 | 4047 | 0.96 | 121 | 3.6 |
| PRO _s (mg kg ⁻¹ grape) | 1122 | 781 | 1351 | 0.81 | 113 | 10.1 |
| FRV _s (mg kg ⁻¹ grape) | 491 | 359 | 610 | 0.78 | 48 | 9.7 |
| C (mg kg ⁻¹ grape) | 81.0 | 57.6 | 126.6 | 0.28 | 20.0 | 24.6 |
| EC (mg kg ⁻¹ grape) | 46.0 | 34.1 | 63.3 | 0.46 | 7.1 | 15.4 |
| ECG (mg kg ⁻¹ grape) | 2.3 | 1.3 | 3.6 | 0.70 | 0.5 | 22.2 |
| TM (mg kg ⁻¹ grape) | 130.0 | 95.3 | 193.5 | 0.18 | 27.3 | 21.0 |
| B ₁ (mg kg ⁻¹ grape) | 20.5 | 13.8 | 29.9 | 0.70 | 3.3 | 16.2 |
| B ₂ (mg kg ⁻¹ grape) | 17.9 | 11.7 | 23.7 | 0.87 | 1.9 | 10.5 |
| TP (mg kg ⁻¹ grape) | 316.9 | 207.4 | 449.0 | 0.63 | 51.9 | 16.4 |
| mDP | 5.9 | 4.9 | 7.5 | 0.56 | 0.6 | 10.8 |
| G% | 6.8 | 5.4 | 8.9 | 0.49 | 0.8 | 12.2 |
| A _{s,280} g ⁻¹ seed | 0.436 | 0.324 | 0.567 | 0.85 | 0.038 | 8.7 |
| TF _s (mg g ⁻¹ seed) | 82.9 | 69.9 | 103.8 | 0.86 | 5.7 | 6.9 |
| PRO _s (mg g ⁻¹ seed) | 28.1 | 21.1 | 35.7 | 0.68 | 3.3 | 11.8 |
| FRV _s (mg g ⁻¹ seed) | 12.3 | 9.6 | 15.9 | 0.56 | 1.5 | 11.9 |
| C (mg g ⁻¹ seed) | 2.05 | 1.41 | 3.15 | 0.66 | 0.41 | 19.7 |
| EC (mg g ⁻¹ seed) | 1.16 | 0.96 | 1.57 | 0.19 | 0.16 | 13.7 |
| ECG (mg g ⁻¹ seed) | 0.06 | 0.03 | 0.09 | 0.61 | 0.01 | 22.9 |
| TM (mg g ⁻¹ seed) | 3.35 | 2.42 | 4.81 | 0.66 | 0.49 | 14.5 |
| B ₁ (mg g ⁻¹ seed) | 0.50 | 0.34 | 0.66 | 0.99 | 0.01 | 2.5 |
| B ₂ (mg g ⁻¹ seed) | 0.44 | 0.31 | 0.55 | 0.57 | 0.07 | 14.9 |
| TP (mg g ⁻¹ seed) | 7.81 | 5.54 | 10.26 | 0.52 | 1.02 | 13.0 |
| <i>2011 vintage^a</i> | | | | | | |
| A _{s,280} kg ⁻¹ grape | 14.8 | 10.2 | 21.2 | 0.49 | 2.8 | 19.1 |
| TF _s (mg kg ⁻¹ grape) | 1538 | 1240 | 1990 | 0.90 | 101 | 6.6 |
| PRO _s (mg kg ⁻¹ grape) | 732 | 492 | 1072 | 0.48 | 149 | 20.3 |
| FRV _s (mg kg ⁻¹ grape) | 731 | 405 | 1322 | 0.86 | 110 | 15.0 |
| C (mg kg ⁻¹ grape) | 91.7 | 59.7 | 140.8 | 0.73 | 14.4 | 15.7 |
| EC (mg kg ⁻¹ grape) | 48.6 | 28.9 | 72.4 | 0.93 | 4.8 | 10.0 |
| ECG (mg kg ⁻¹ grape) | 1.5 | 0.9 | 2.8 | 0.79 | 0.3 | 19.0 |
| TM (mg kg ⁻¹ grape) | 140.9 | 97.1 | 205.2 | 0.61 | 23.6 | 16.8 |

| | | | | | | |
|--|-------|-------|-------|------|-------|------|
| B ₁ (mg kg ⁻¹ grape) | 18.8 | 14.1 | 28.0 | 0.45 | 3.2 | 16.9 |
| B ₂ (mg kg ⁻¹ grape) | 19.4 | 12.6 | 26.7 | 0.73 | 2.9 | 15.1 |
| TP (mg kg ⁻¹ grape) | 299.5 | 137.8 | 436.1 | 0.60 | 67.7 | 22.6 |
| mDP | 5.3 | 4.4 | 6.1 | 0.27 | 0.5 | 8.9 |
| G% | 7.4 | 4.3 | 11.4 | 0.94 | 0.6 | 8.8 |
| A _{s,280} g ⁻¹ seed | 0.389 | 0.241 | 0.542 | 0.55 | 0.087 | 22.3 |
| TF _s (mg g ⁻¹ seed) | 41.9 | 32.4 | 54.7 | 0.80 | 4.9 | 11.6 |
| PRO _s (mg g ⁻¹ seed) | 21.1 | 12.5 | 29.3 | 0.27 | 5.2 | 24.9 |
| FRV _s (mg g ⁻¹ seed) | 21.0 | 10.3 | 41.4 | 0.98 | 1.6 | 7.6 |
| C (mg g ⁻¹ seed) | 2.51 | 1.52 | 3.50 | 0.91 | 0.24 | 9.5 |
| EC (mg g ⁻¹ seed) | 1.31 | 0.85 | 2.07 | 0.40 | 0.30 | 22.7 |
| ECG (mg g ⁻¹ seed) | 0.04 | 0.03 | 0.07 | 0.99 | 0.00 | 4.9 |
| TM (mg g ⁻¹ seed) | 3.86 | 2.47 | 5.64 | 0.81 | 0.47 | 12.3 |
| B ₁ (mg g ⁻¹ seed) | 0.52 | 0.35 | 0.73 | 0.88 | 0.06 | 10.8 |
| B ₂ (mg g ⁻¹ seed) | 0.53 | 0.35 | 0.76 | 0.46 | 0.12 | 21.7 |
| TP (mg g ⁻¹ seed) | 8.20 | 3.37 | 12.31 | 0.35 | 2.34 | 28.5 |

2010 and 2011 *vintages*^b

| | | | | | | |
|--|-------|-------|-------|------|-------|------|
| A _{s,280} kg ⁻¹ grape | 16.2 | 10.2 | 23.0 | 0.85 | 1.8 | 11.3 |
| TF _s (mg kg ⁻¹ grape) | 2308 | 1240 | 3796 | 0.94 | 327 | 14.2 |
| PRO _s (mg kg ⁻¹ grape) | 923 | 492 | 1351 | 0.89 | 118 | 12.7 |
| FRV _s (mg kg ⁻¹ grape) | 630 | 359 | 1322 | 0.97 | 51 | 8.2 |
| C (mg kg ⁻¹ grape) | 87.2 | 57.6 | 126.6 | 0.53 | 16.5 | 18.9 |
| EC (mg kg ⁻¹ grape) | 47.4 | 28.9 | 72.4 | 0.10 | 11.1 | 23.5 |
| ECG (mg kg ⁻¹ grape) | 1.9 | 0.9 | 3.6 | 0.72 | 0.5 | 23.8 |
| TM (mg kg ⁻¹ grape) | 134.8 | 95.3 | 205.2 | 0.35 | 27.3 | 20.2 |
| B ₁ (mg kg ⁻¹ grape) | 19.5 | 13.8 | 29.9 | 0.63 | 3.1 | 15.9 |
| B ₂ (mg kg ⁻¹ grape) | 18.5 | 11.7 | 26.7 | 0.63 | 3.1 | 16.4 |
| TP (mg kg ⁻¹ grape) | 302.3 | 137.8 | 449.0 | 0.67 | 51.7 | 17.1 |
| mDP | 5.6 | 4.4 | 6.9 | 0.43 | 0.6 | 10.1 |
| G% | 7.2 | 4.3 | 11.4 | 0.87 | 0.8 | 10.5 |
| A _{s,280} g ⁻¹ seed | 0.420 | 0.241 | 0.567 | 0.65 | 0.072 | 17.1 |
| TF _s (mg g ⁻¹ seed) | 62.3 | 33.0 | 103.8 | 0.95 | 7.2 | 11.5 |
| PRO _s (mg g ⁻¹ seed) | 23.9 | 12.5 | 35.7 | 0.83 | 3.6 | 15.1 |
| FRV _s (mg g ⁻¹ seed) | 15.4 | 9.6 | 23.9 | 0.93 | 1.7 | 11.1 |
| C (mg g ⁻¹ seed) | 2.29 | 1.41 | 3.50 | 0.73 | 0.40 | 17.4 |
| EC (mg g ⁻¹ seed) | 1.22 | 0.85 | 2.07 | 0.44 | 0.26 | 21.5 |
| ECG (mg g ⁻¹ seed) | 0.05 | 0.03 | 0.09 | 0.90 | 0.01 | 13.8 |
| TM (mg g ⁻¹ seed) | 3.59 | 2.42 | 5.64 | 0.65 | 0.61 | 16.9 |

| | | | | | | |
|--|------|------|-------|------|------|------|
| B ₁ (mg g ⁻¹ seed) | 0.52 | 0.34 | 0.73 | 0.43 | 0.09 | 17.3 |
| B ₂ (mg g ⁻¹ seed) | 0.49 | 0.31 | 0.76 | 0.40 | 0.11 | 22.3 |
| TP (mg g ⁻¹ seed) | 7.88 | 5.48 | 11.30 | 0.62 | 1.14 | 14.4 |

^an = 42. ^bn = 84. ^cR_c = correlation coefficient of calibration, ^dSEC = standard error of calibration, ^eSEC% = (SEC/Mean) x 100.

Table 3. Validation of NIR Calibration Models Developed for Absorbance at 280 nm and Extractable Content of Phenolic Compounds in Intact Nebbiolo Seeds Using Different Number of Vintages

| Chemical parameter | Mean | Minimum | Maximum | R_v^c | SEP ^d | SEP% ^e | RPD ^f | RPIQ ^g |
|--|-------|---------|---------|---------|------------------|-------------------|------------------|-------------------|
| <i>2010 vintage^a</i> | | | | | | | | |
| $A_{s,280}$ kg ⁻¹ grape | 17.3 | 13.6 | 21.0 | 0.75 | 1.8 | 10.4 | 1.5 | 2.7 |
| TF _s (mg kg ⁻¹ grape) | 3345 | 2826 | 3901 | 0.94 | 95 | 2.8 | 4.0 | 6.1 |
| PRO _s (mg kg ⁻¹ grape) | 1167 | 871 | 1310 | 0.88 | 119 | 10.2 | 1.5 | 2.6 |
| FRV _s (mg kg ⁻¹ grape) | 502 | 398 | 597 | 0.84 | 46 | 9.1 | 1.8 | 3.5 |
| C (mg kg ⁻¹ grape) | 88.0 | 57.6 | 124.0 | 0.29 | 20.9 | 23.7 | 1.0 | 1.8 |
| EC (mg kg ⁻¹ grape) | 47.5 | 34.1 | 57.7 | 0.35 | 6.4 | 13.5 | 1.0 | 1.6 |
| ECG (mg kg ⁻¹ grape) | 2.3 | 1.3 | 2.8 | 0.71 | 0.3 | 12.6 | 1.3 | 2.1 |
| TM (mg kg ⁻¹ grape) | 137.6 | 100.0 | 184.2 | 0.42 | 30.1 | 21.9 | 1.0 | 1.7 |
| B ₁ (mg kg ⁻¹ grape) | 19.9 | 13.8 | 23.8 | 0.66 | 2.8 | 14.1 | 1.0 | 1.2 |
| B ₂ (mg kg ⁻¹ grape) | 17.2 | 12.6 | 23.1 | 0.86 | 1.9 | 11.0 | 1.9 | 2.9 |
| TP (mg kg ⁻¹ grape) | 305.4 | 263.5 | 386.5 | 0.81 | 33.9 | 11.1 | 1.4 | 1.9 |
| mDP | 5.9 | 5.4 | 6.7 | 0.42 | 0.5 | 8.6 | 1.1 | 1.5 |
| G% | 7.2 | 5.9 | 8.5 | 0.55 | 0.8 | 10.9 | 1.1 | 2.0 |
| $A_{s,280}$ g ⁻¹ seed | 0.451 | 0.402 | 0.523 | 0.71 | 0.039 | 8.6 | 1.3 | 2.2 |
| TF _s (mg g ⁻¹ seed) | 83.8 | 74.4 | 97.2 | 0.82 | 6.2 | 7.4 | 1.4 | 2.6 |
| PRO _s (mg g ⁻¹ seed) | 29.1 | 22.1 | 33.3 | 0.81 | 3.0 | 10.2 | 1.5 | 2.6 |
| FRV _s (mg g ⁻¹ seed) | 12.4 | 9.9 | 14.7 | 0.72 | 1.5 | 11.8 | 1.3 | 2.3 |
| C (mg g ⁻¹ seed) | 2.12 | 1.41 | 2.46 | 0.63 | 0.37 | 17.3 | 0.9 | 1.6 |
| EC (mg g ⁻¹ seed) | 1.14 | 0.96 | 1.30 | 0.01 | 0.13 | 11.8 | 1.0 | 1.7 |
| ECG (mg g ⁻¹ seed) | 0.05 | 0.03 | 0.07 | 0.68 | 0.01 | 16.7 | 1.3 | 2.4 |
| TM (mg g ⁻¹ seed) | 3.16 | 2.47 | 4.02 | 0.61 | 0.52 | 16.5 | 1.1 | 1.6 |
| B ₁ (mg g ⁻¹ seed) | 0.51 | 0.34 | 0.60 | 0.76 | 0.05 | 8.9 | 1.3 | 1.9 |
| B ₂ (mg g ⁻¹ seed) | 0.44 | 0.31 | 0.53 | 0.70 | 0.06 | 12.6 | 1.4 | 2.1 |
| TP (mg g ⁻¹ seed) | 7.73 | 5.76 | 9.79 | 0.74 | 1.05 | 13.6 | 1.3 | 2.8 |
| <i>2011 vintage^a</i> | | | | | | | | |
| $A_{s,280}$ kg ⁻¹ grape | 14.2 | 11.9 | 18.3 | 0.46 | 2.2 | 15.3 | 1.1 | 2.0 |
| TF _s (mg kg ⁻¹ grape) | 1577 | 1357 | 1777 | 0.88 | 105 | 6.7 | 1.7 | 3.4 |
| PRO _s (mg kg ⁻¹ grape) | 763 | 553 | 926 | 0.74 | 107 | 14.1 | 1.3 | 2.3 |
| FRV _s (mg kg ⁻¹ grape) | 744 | 600 | 845 | 0.91 | 98 | 13.2 | 1.0 | 1.9 |
| C (mg kg ⁻¹ grape) | 92.5 | 61.7 | 120.8 | 0.68 | 16.9 | 18.2 | 1.3 | 2.4 |
| EC (mg kg ⁻¹ grape) | 46.4 | 34.5 | 57.8 | 0.91 | 4.1 | 8.9 | 2.3 | 4.3 |
| ECG (mg kg ⁻¹ grape) | 1.6 | 1.1 | 2.3 | 0.77 | 0.3 | 18.9 | 1.4 | 2.7 |
| TM (mg kg ⁻¹ grape) | 142.9 | 103.6 | 195.5 | 0.65 | 25.5 | 17.8 | 1.3 | 2.3 |

| | | | | | | | | |
|--|-------|-------|-------|------|-------|------|-----|-----|
| B ₁ (mg kg ⁻¹ grape) | 18.4 | 14.8 | 24.2 | 0.45 | 3.1 | 17.0 | 1.1 | 1.8 |
| B ₂ (mg kg ⁻¹ grape) | 19.2 | 15.1 | 25.3 | 0.49 | 3.5 | 18.2 | 1.1 | 1.9 |
| TP (mg kg ⁻¹ grape) | 313.5 | 223.3 | 396.1 | 0.61 | 49.2 | 15.7 | 1.3 | 2.0 |
| mDP | 5.4 | 4.4 | 6.0 | 0.28 | 0.4 | 7.9 | 1.0 | 2.2 |
| G% | 7.3 | 5.0 | 8.6 | 0.95 | 0.6 | 8.0 | 2.4 | 3.9 |
| A _{s,280} g ⁻¹ seed | 0.409 | 0.294 | 0.519 | 0.53 | 0.078 | 19.0 | 1.2 | 2.5 |
| TF _s (mg g ⁻¹ seed) | 44.2 | 35.1 | 53.4 | 0.76 | 5.1 | 11.5 | 1.5 | 2.7 |
| PRO _s (mg g ⁻¹ seed) | 19.8 | 15.1 | 26.4 | 0.23 | 4.0 | 20.1 | 1.0 | 1.7 |
| FRV _s (mg g ⁻¹ seed) | 18.6 | 14.0 | 23.5 | 0.91 | 1.8 | 9.9 | 1.7 | 2.3 |
| C (mg g ⁻¹ seed) | 2.49 | 1.87 | 3.25 | 0.93 | 0.18 | 7.1 | 2.8 | 5.8 |
| EC (mg g ⁻¹ seed) | 1.30 | 0.96 | 1.85 | 0.57 | 0.29 | 22.3 | 1.2 | 2.3 |
| ECG (mg g ⁻¹ seed) | 0.04 | 0.03 | 0.05 | 0.84 | 0.01 | 16.2 | 1.3 | 2.5 |
| TM (mg g ⁻¹ seed) | 3.83 | 2.80 | 5.15 | 0.68 | 0.59 | 15.3 | 1.3 | 2.3 |
| B ₁ (mg g ⁻¹ seed) | 0.49 | 0.38 | 0.68 | 0.80 | 0.07 | 15.1 | 1.4 | 3.1 |
| B ₂ (mg g ⁻¹ seed) | 0.52 | 0.38 | 0.73 | 0.39 | 0.11 | 21.4 | 1.1 | 1.8 |
| TP (mg g ⁻¹ seed) | 8.75 | 5.93 | 11.79 | 0.53 | 1.83 | 20.9 | 1.2 | 1.6 |

2010 and 2011 *vintages*^b

| | | | | | | | | |
|--|-------|-------|-------|------|-------|------|-----|-----|
| A _{s,280} kg ⁻¹ grape | 15.7 | 11.9 | 21.0 | 0.82 | 1.8 | 11.2 | 1.7 | 2.7 |
| TF _s (mg kg ⁻¹ grape) | 2556 | 1357 | 4047 | 0.94 | 309 | 12.1 | 3.0 | 6.0 |
| PRO _s (mg kg ⁻¹ grape) | 958 | 568 | 1279 | 0.89 | 121 | 12.6 | 2.1 | 4.3 |
| FRV _s (mg kg ⁻¹ grape) | 604 | 395 | 828 | 0.87 | 74 | 12.3 | 2.1 | 3.2 |
| C (mg kg ⁻¹ grape) | 84.8 | 60.6 | 115.8 | 0.51 | 16.7 | 19.7 | 1.2 | 2.3 |
| EC (mg kg ⁻¹ grape) | 47.1 | 38.1 | 57.7 | 0.10 | 6.5 | 13.8 | 1.0 | 1.7 |
| ECG (mg kg ⁻¹ grape) | 1.8 | 1.1 | 2.8 | 0.69 | 0.4 | 23.4 | 1.4 | 2.4 |
| TM (mg kg ⁻¹ grape) | 141.9 | 102.3 | 195.5 | 0.36 | 27.5 | 19.4 | 1.1 | 1.5 |
| B ₁ (mg kg ⁻¹ grape) | 19.3 | 14.8 | 26.7 | 0.52 | 3.1 | 16.1 | 1.1 | 1.8 |
| B ₂ (mg kg ⁻¹ grape) | 18.5 | 11.9 | 25.5 | 0.67 | 3.2 | 17.5 | 1.2 | 2.2 |
| TP (mg kg ⁻¹ grape) | 321.8 | 208.2 | 436.1 | 0.65 | 55.9 | 17.4 | 1.2 | 2.0 |
| mDP | 5.4 | 4.8 | 6.5 | 0.48 | 0.4 | 8.1 | 1.1 | 1.8 |
| G% | 7.1 | 5.0 | 8.9 | 0.72 | 0.9 | 12.0 | 1.4 | 2.1 |
| A _{s,280} g ⁻¹ seed | 0.409 | 0.294 | 0.519 | 0.67 | 0.048 | 11.7 | 1.4 | 1.6 |
| TF _s (mg g ⁻¹ seed) | 60.6 | 32.4 | 88.3 | 0.91 | 8.8 | 14.5 | 2.4 | 5.0 |
| PRO _s (mg g ⁻¹ seed) | 25.4 | 15.7 | 32.6 | 0.75 | 3.7 | 14.7 | 1.4 | 2.2 |
| FRV _s (mg g ⁻¹ seed) | 15.8 | 11.0 | 22.7 | 0.85 | 2.1 | 13.4 | 1.8 | 2.6 |
| C (mg g ⁻¹ seed) | 2.31 | 1.50 | 3.25 | 0.62 | 0.38 | 16.6 | 1.2 | 1.9 |
| EC (mg g ⁻¹ seed) | 1.26 | 1.02 | 1.57 | 0.46 | 0.16 | 12.9 | 1.1 | 1.7 |
| ECG (mg g ⁻¹ seed) | 0.05 | 0.03 | 0.07 | 0.85 | 0.01 | 14.7 | 1.8 | 2.7 |
| TM (mg g ⁻¹ seed) | 3.57 | 2.60 | 5.05 | 0.56 | 0.58 | 16.2 | 1.2 | 1.7 |

| | | | | | | | | |
|--|------|------|-------|------|------|------|-----|-----|
| B ₁ (mg g ⁻¹ seed) | 0.49 | 0.35 | 0.68 | 0.47 | 0.09 | 17.8 | 1.1 | 1.7 |
| B ₂ (mg g ⁻¹ seed) | 0.48 | 0.31 | 0.75 | 0.40 | 0.10 | 19.7 | 1.1 | 0.9 |
| TP (mg g ⁻¹ seed) | 8.22 | 5.54 | 11.31 | 0.67 | 1.32 | 16.1 | 1.3 | 1.7 |

^an = 18. ^bn = 36. ^cR_v = correlation coefficient of validation, ^dSEP = standard error of prediction, ^eSEP% = (SEP/Mean) x 100, ^fRPD = residual predictive deviation (SD/SEP), SD = standard deviation, ^gRPIQ = IQ/SEP, IQ = interquartile amplitude.

Table 4. Absorbance at 280 nm and Extractable Content of Phenolic Compounds in Intact Nebbiolo Seeds from the Three Growing Zones Studied in 2011

| Chemical parameter ^a | Growing zone | | | Sign ^d |
|--|-------------------------|-----------------------|-----------------------|-------------------|
| | Valtellina ^b | Piedmont ^c | Trentino ^c | |
| A _{s,280} kg ⁻¹ grape | 14.6±3.0 | 20.2±1.8 | 20.1±1.7 | ns |
| TF _s (mg kg ⁻¹ grape) | 1550±211 | 1357±152 | 1261±124 | ns |
| PRO _s (mg kg ⁻¹ grape) | 754±158 ^b | 1119±43 ^a | 1135±129 ^a | *** |
| FRV _s (mg kg ⁻¹ grape) | 735±185 | 1119±124 | 1072±72 | ns |
| A _{s,280} g ⁻¹ seed | 0.400±0.092 | 0.549±0.021 | 0.425±0.054 | ns |
| TF _s (mg g ⁻¹ seed) | 42.6±7.8 | 36.8±0.6 | 26.7±3.9 | ns |
| PRO _s (mg g ⁻¹ seed) | 20.7±5.0 | 30.5±2.7 | 24.1±3.9 | ns |
| FRV _s (mg g ⁻¹ seed) | 20.3±6.6 | 30.3±0.8 | 22.7±2.6 | ns |

^aAll data are expressed as average value ± standard deviation. ^bn = 60. ^cn = 9. ^dSign: *** and ns indicate significance at p < 0.001 and not significant, respectively. Different Latin letters within the same row indicate significant differences among growing zones for each chemical parameter (*Tukey-b test*; p < 0.01).

Table 5. Loadings of Principal Components for NIR Spectral Data of Intact Nebbiolo Seeds from the Three Growing Zones Studied in 2011

| Principal component | Valtellina ^a | Piedmont ^b | Trentino ^b | Sign ^c |
|---------------------|-------------------------|-----------------------|-----------------------|-------------------|
| 1 | -0.0970 ^a | 0.7226 ^b | 0.4574 ^b | *** |
| 2 | 0.0071 ^b | 0.3601 ^c | -0.5134 ^a | *** |
| 3 | 0.0367 | 0.1148 | 0.1505 | ns |
| 4 | -0.0163 | -0.1196 | -0.1703 | ns |

^an = 40. ^bn = 3. ^cSign: *** and ns indicate significance at p < 0.001 and not significant, respectively. Different Latin letters within the same row indicate significant differences among growing zones for each principal component (*Tukey-b test*; p < 0.01).

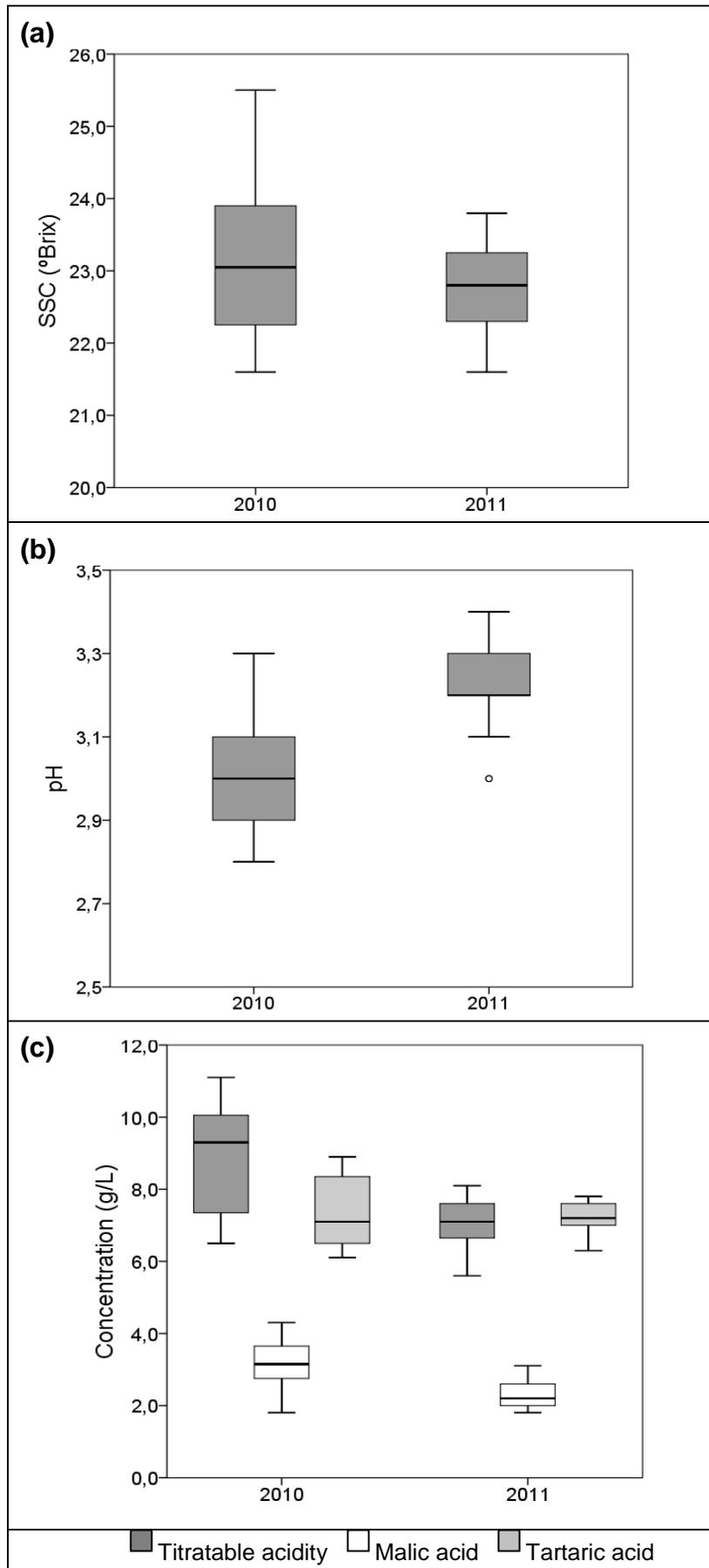


Figure 1

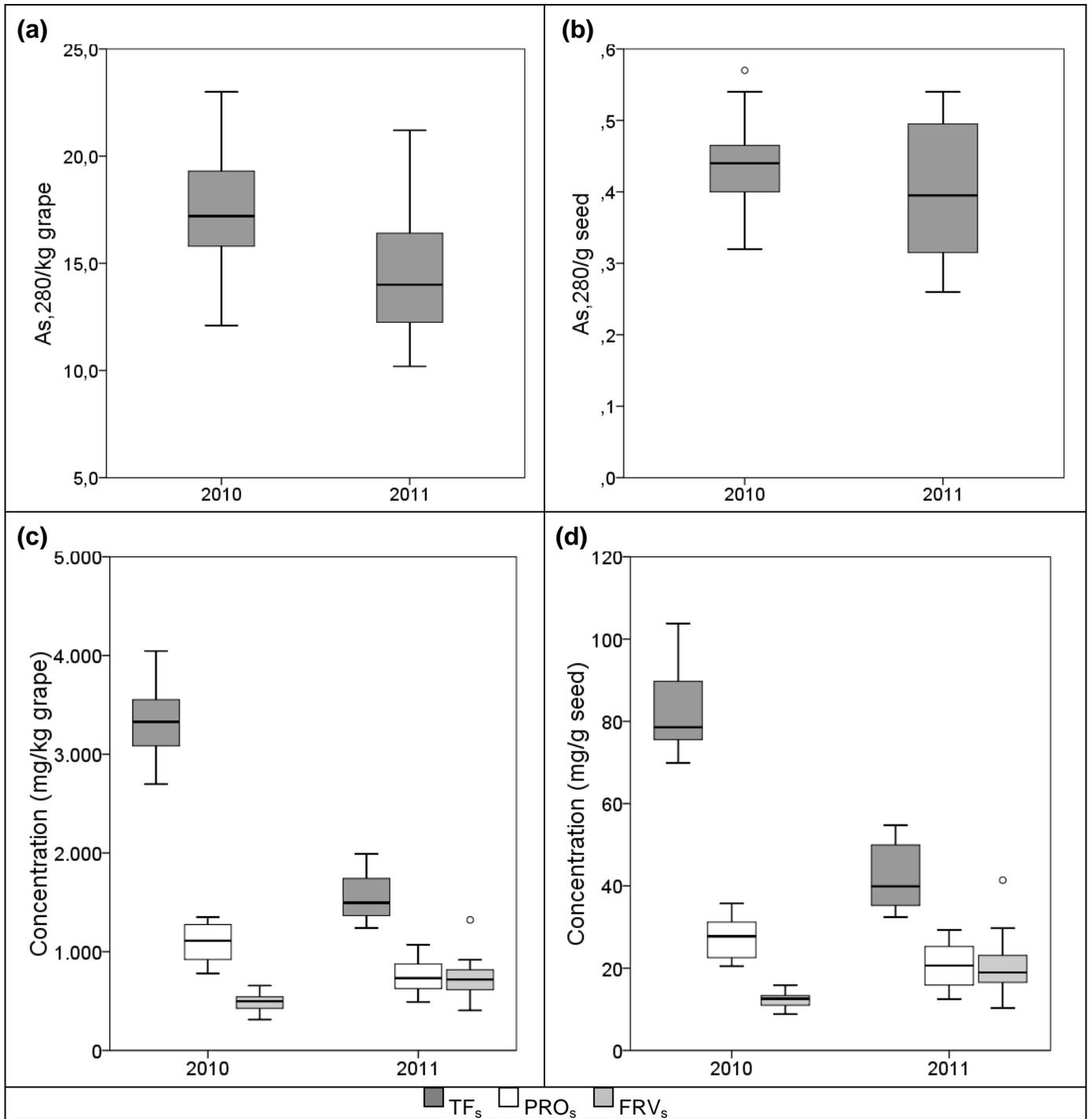


Figure 2

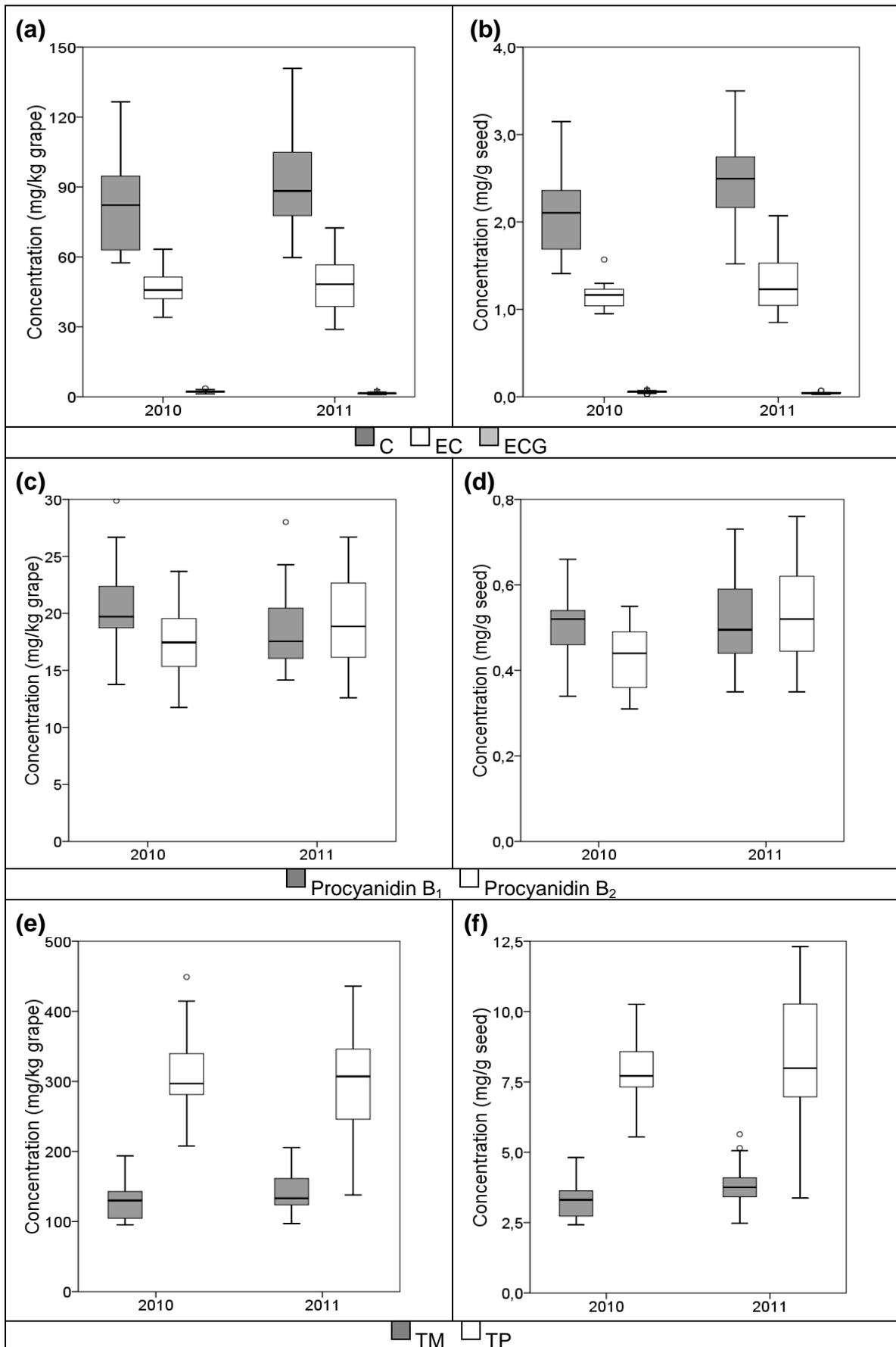


Figure 3

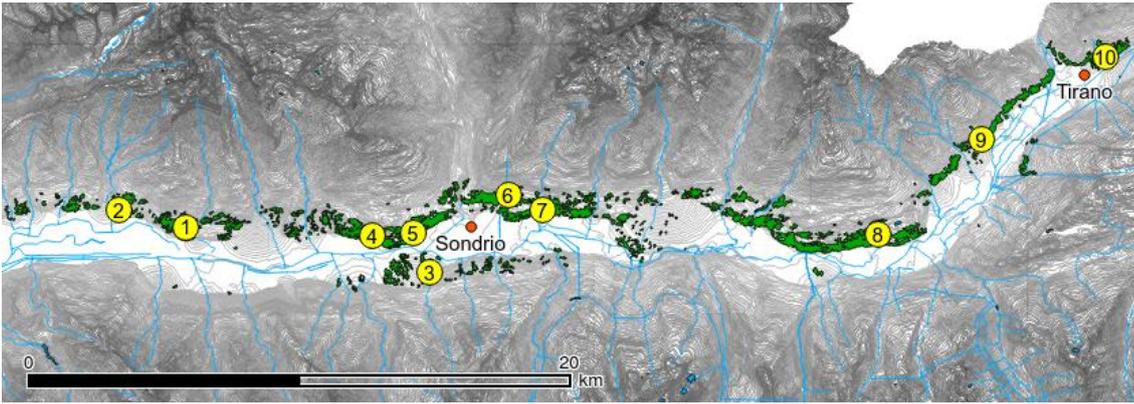


Figure S1. Geographical distribution of vineyards (green) in the Valtellina valley, with the selected vineyard zones marked (yellow, see Table S1 for the vineyards information). Terrain data provided by Regione Lombardia (Italy).

Table S1. Location and Geographical Coordinates of the Different Vineyard Zones Studied

| Growing zone | Vineyard no. | Location | North coordinate | East coordinate |
|---------------------|---------------------|--------------------------|-------------------------|------------------------|
| | 1 | Berbenno | 46.1703 | 9.7373 |
| | 2 | Maroggia | 46.1764 | 9.7063 |
| | 3 | Albosaggia | 46.1544 | 9.8530 |
| | 4 | Triasso | 46.1671 | 9.8263 |
| Valtellina | 5 | Sassella | 46.1673 | 9.8457 |
| | 6 | Grumello | 46.1799 | 9.8918 |
| | 7 | Inferno | 46.1747 | 9.9071 |
| | 8 | Valgella | 46.1653 | 10.0679 |
| | 9 | Bianzone | 46.1961 | 10.1182 |
| | 10 | Tirano | 46.2234 | 10.1775 |
| Piedmont | | Grinzane Cavour | 44.6527 | 7.9950 |
| Trentino | | San Michele all'Adige | 46.1940 | 11.1371 |

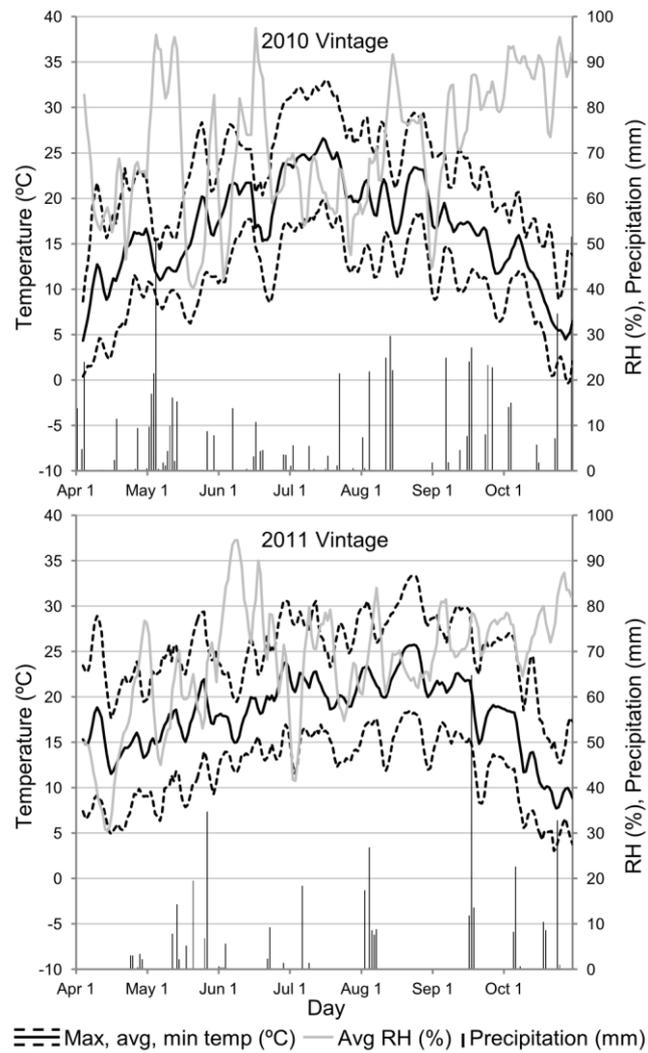


Figure S2. Climatic conditions of 2010 and 2011 vintages in terms of maximum, average and minimum daily temperature, average daily relative humidity (RH) and total daily precipitations during the vegetative period of the vine.