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Nondestructive Diagnostic Test for Nitrogen Nutrition of Grapevine (*Vitis vinifera* L.) Based on Dualex Leaf-Clip Measurements in the Field

Zoran G. Cerovic,*[,†,‡] Naïma Ben Ghozlen,[§] Charlotte Milhade,[§] Mickaël Obert,[∥] Sébastien Debuisson,[⊥] and Marine Le Moigne[§]

[†]Laboratoire Écologie Systématique et Évolution, Université Paris-Sud, UMR 8079 CNRS, Bâtiment 362, F-91405 Orsay, France

[‡]AgroParisTech, F-75231 Paris, France

[§]FORCE-A, Université Paris-Sud, Bâtiment 503, F-91405 Orsay, France

[∥]Château Gazin, Chemin de Chantecaille, F-33500 Pomerol, France

[⊥]Comité Interprofessionnel du Vin de Champagne (CIVC), 5 rue Henri-Martin, boîte postale 135, F-51200 Epernay, France

Supporting Information

ABSTRACT: Crop nitrogen status is a major issue for crop yield and quality. It is usually assessed by destructive leaf or petiole tissue analysis. A quantitative nondestructive optical estimation of N sufficiency would be a great leap forward toward precision crop management. We therefore calibrated three optical indices against leaf nitrogen content: chlorophyll (Chl), epidermal flavonols, and the nitrogen balance index (NBI), which is the ratio of the former two indices. NBI was the best estimator of leaf N content measured by the Dumas or Kjeldahl method with a root-mean-square error smaller than 2 mg of N g^{-1} dry weight, followed by Chl (3 mg g^{-1}) and flavonols (4 mg g^{-1}). This allowed us to propose the threshold values for the Dualex optical indices that characterize nitrogen supply to grapevines: the first is the threshold below which N supply to the vine can be considered deficient, and the second is the threshold above which N supply is excessive. For a putative optimal N content of 30 mg g^{-1} < x < 40 mg g^{-1}, these thresholds are 30 μg cm^{-2} < x < 40 μg cm^{-2} for Chl and 11 < x < 18 for NBI at flowering. At bunch closure, for N thresholds of 22 < x < 32, Chl is 29 < x < 37 and NBI is 8 < x < 11, in respective units. These values should be verified and refined in the future for various growth regions and cultivars using the specified protocol. The sample size should be 36−60 leaves from a fixed node position, preferably node no. 5 from the tip of the shoot. An alternative to the use of the NBI would be to discard leaves that are not light exposed by checking their flavonol content and to deduce the N sufficiency directly from the Chl values.

KEYWORDS: *chlorophyll, Dualex, flavonols, nitrogen balance index, nitrogen fertilization, optical sensors, precision farming*

INTRODUCTION

Nitrogen management is a major issue in agriculture.[1] The knowledge of the level of sufficiency or deficiency at the time of potential application is crucial. This is even more the case in precision agriculture where spatial heterogeneity is also taken into account.[2] Crop nitrogen status is usually assessed using destructive leaf or petiole tissue analysis.[3] Leaf N content is an important individual crop trait, usually assessed at the crop population level. It is often used as a predictor (proxy) for the optimization of nitrogen fertilization for maximal yield, or in the case of grapevine (*Vitis vinifera* L.) also for a high grape quality.[4] The choice of the representative grapevine leaf of the whole plant is of paramount importance for both destructive[3] and nondestructive optical tissue testing. Leaf N content changes over the course of plant growth.[5,6] This is why the phenological stage for N estimation must be rigorously defined and respected.

There have been attempts in major grape-producing countries, France, Spain, Italy, Australia, United States, Germany, and South Africa, to propose a norm for leaf N content to help local viticultural practices (for references see the Results and Discussion). For example, in the United States, leaf petiole N content is more frequently analyzed than leaf blade N content.[7,8] Nevertheless, sampling leaf blades may be more accurate for assessing N levels than using leaf petioles.[9] Compared to tissue testing, optical methods provide much faster assessment of crop status and can be extended even to on-the-go fertilization. Optical methods use the major symptoms of nitrogen deficiency, that is, light green leaf color and older leaves turning yellow,[3] but in a much more objective and quantitative way. They are based on leaf transmittance, reflectance, or fluorescence.[10] The most precise optical technique is the use of chlorophyll (Chl) meter leaf clips.[10] The ones most often used are the SPAD-502 from Minolta-Konica and the N-tester, which is a simplified variant introduced for grain crop N management by the Yara Co. The possibility to replace wet chemistry analysis of the grapevine leaf N by the SPAD-502[11,12] or N-tester[13,14] measurements was tested, but Brunetto et al.[15] concluded a low accuracy of the

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SPAD-502 readings in estimating leaf N. The latter might be the consequence of surface-based Chl often compared to mass-based N,[11,15] although the effect of leaf mass per area (LMA) on this relationship has been clearly demonstrated for ligneous species in general[16] or specifically for grapevine.[17] It should be mentioned here that grapevine was also used as a model plant to analyze the potential of optical leaf clips to nondestructively assess leaf Chl content,[18,19] independently of its relation to nitrogen.

Two major problems with the use of the SPAD-502 (N-tester) or equivalent leaf clips are their nonlinearity,[19] especially at optimal and supraoptimal N contents, and the influence of the variable LMA.[20] This sensor nonlinearity is independent of the additional nonlinearity due to the physiological saturation of leaf response to N.[21,22] Recently, in addition to single-parameter Chl leaf clips, both multiparameter leaf clips and multiparameter proximal sensors have become available.[21−23] Therefore, a bi- or multiparametric approach to estimating N can be attempted. Cartelat et al.[24] proposed the use of the nitrogen balance index (NBI), which is the ratio of Chl to epidermal flavonol (Flav) leaf content, for nitrogen nutrition of wheat in the context of precision agriculture. Ten years later, the advent of the new Dualex leaf clip and the proximal sensor Multiplex from FORCE-A has allowed for an easier assessment of this new nitrogen index and has permitted its extension to other crops, including both monocots[21,22,25,26] and dicots.[27,28] Nevertheless, there is a need for a proper quantitative calibration of this index to implement its use in crop diagnostics. Leaf Chl and flavonol contents on a surface basis are both dependent on leaf age and light experienced during growth.[29,30] They both increase with age and light during the first part of the season. During the second part, Chl tends to decrease while Flav remains constantly high.[29] The NBI index (Chl/Flav) is much less sensitive to phenology, and it reflects the N availability better than either of the two indicators used individually.[16,21,22,24,25,31,32]

Most important experiments on N fertilization were performed under controlled conditions in potted plants.[33−35] We wanted to test the NBI approach in vineyards under real viticultural practices for which this optical diagnostic approach was originally designed. There are no published calibrations for N measurements by these new devices, namely, the Dualex and the Multiplex. As the Dualex responds linearly to both Chl and Flav leaf contents,[19] the NBI index can potentially be quantitatively related to leaf N content. Therefore, the objectives of this paper are (1) to provide a calibration of the Dualex response to leaf N content, (2) to verify the robustness of the N estimation by NBI in grapevine as a diagnostic method, and (3) to propose a protocol for leaf sampling for optical diagnostics of N content in grapevine.

MATERIALS AND METHODS

Experimental Design and Sensors. Experiments were performed over a period of 5 years, parallel to the development of the new Dualex leaf clips. Therefore, both the SPAD-502 (Konica-Minolta, Tokyo, Japan) and the Dualex 4 Scientific (FORCE-A, Orsay, France) were used for the Chl measurements. SPAD-502 units were transformed into leaf Chl content on a surface basis (μg cm^{-2}) using the consensus formula from Cerovic et al.[19] The Dualex 4 Scientific Chl was used as given by the device because it was calibrated in μg cm^{-2} units.[19] Epidermal Flav values were measured by the Dualex leaf-clip version Dualex 3.3 (FORCE-A) and the version Dualex 4 Scientific. Absorbance in the UV at 375 nm due to flavonol presence in the epidermis was used as given by the device without transformation.

For the data set Leaves 2007, leaves were sampled at the Fort Chabrol vineyard in Epernay, Champagne, France (03°57′ E, 49°02′ N) in May 2007 just before flowering (BBCH 57).[36] No. 5 leaves counted from the tip of the shoots were chosen from Pinot noir vines. The first leaf having a central vein longer than 3 cm was considered as leaf no. 1. Six light-exposed and six shaded leaves were measured with the SPAD-502 from the abaxial leaf side once on the left-hand side (position 1) and once on the right-hand side (position 2) from the main vein (cf. Figure S2, Supporting Information). Total Chl for each spot was calculated from the SPAD-502 measurements using the consensus equation from Cerovic et al.[19] Flavonols were measured by the Dualex 3.3 on the same leaf spots as for Chl, but from both the adaxial and abaxial leaf sides. The mean Chl value from the two spots was calculated for each leaf. The mean Flav value was calculated for the two spots from the sum of the abaxial and adaxial sides. The NBI[24,37] was calculated from these mean Chl and Flav data for each leaf. A 1 cm^2 disk sampled from the spot in position 1 was used for Chl extraction and a 1 cm^2 disk sampled from the spot in position 2 for nitrogen content analysis.

For the Leaves 2009 data set, leaves were sampled on May 28 and 29, 2009, just before flowering (BBCH 57) at the Plumecoq experimental vineyard near Epernay, France (03°58′ E, 49°01′ N) belonging to the Champagne committee. A total of 59 no. 5 leaves counted from the shoot tip were sampled from Pinot noir (29 leaves) and Chardonnay (30 leaves) planted in 1996 and trained to a "Chablis" system, with three canes. Leaf Chl was measured with both the SPAD-502 and the Dualex 4 Scientific on the adaxial leaf side on the two spots, as for the Leaves 2007 data set (cf. Figure S1, Supporting Information). Flavonols were measured by both the Dualex 3.3 and the first version of the Dualex 4 Scientific on the same leaf spots on both leaf sides. The mean Chl value for the two spots was calculated for each leaf, both for Dualex 4 Scientific and for SPAD-502 measurements. The mean Flav value was calculated for the two spots from the sum of the abaxial and adaxial side measurements with the Dualex 3.3. The SPAD-502 measurements were transformed using the consensus equation from Cerovic et al.[19] The NBI index was calculated from the Chl and Flav data for each leaf. A 0.58 cm^2 disk sampled from the spot in position 1 was used for Chl extraction and a 0.58 cm^2 disk sampled from the spot in position 2 for nitrogen content analysis.

For the Plot 2009 data set, 60 leaves were sampled from each of the 27 commercial plots scattered around Epernay in Champagne. In total, 1620 leaves were measured. Plots from 0.3 to 1 ha, having different soils and cultural practices, planted with Pinot noir, Meunier, and Chardonnay of different ages were chosen. No. 5 leaves were sampled from three rows per plot from May 18 to May 27, 2009, just before flowering (BBCH 55−57). Two spots per leaf, as in the Leaves 2007 data set, were measured by the SPAD-502 for Chl and the Dualex 3.3 for Flav. The total Chl for each spot was calculated from the SPAD-502 measurements using the consensus equation from Cerovic et al.[19] The Chl, Flav, and NBI indices were calculated for each leaf as described in the Leaves 2009 data set. Two 0.58 cm^2 disks were sampled per leaf (both spots) for nitrogen content analysis (see below). The averages of the three optical indices, Chl, Flav, and NBI, and the N content were calculated for each plot from the 60-leaf data.

The Subplots 2012 data set was obtained at Chateau Gazin near Bordeaux, France (0°11′ E, 44°55′ N) on July 27, 2012, at the bunch closure stage (BBCH 79). The plot was planted with Merlot noir on SO4 in 1977 and trained as a double Guyot, with 1.25 m intrarow and 1.4 m interrow spacing. A total of 10 subplots were selected on the basis of a map of the NBI produced by a Multiplex-mounted system (FORCE-A) a few days before (cf. Figure S2, Supporting Information). Three leaves from three consecutive vines were measured by the most recent version of the Dualex 4 Scientific for both Chl and Flav. These nine-leaf batches were repeated three times on each of the 10 subplots. Thanks to the relative heterogeneity inside each of the chosen subplots (cf. Figure S1, Supporting Information), the nine-leaf batches could be used individually, producing a final data

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set of 31 samples (3 × 10) + 1, because in the last subplot an extra batch of nine leaves was measured. Although only mature leaves on primary shoots were sampled, as in the three other data sets, there were two major differences: the exact node position on the shoot was not assessed, and only one spot on the leaf was measured; care was taken to only sample leaves from the same height of the canopy at which the Multiplex measurements were made, above the second wire. The details of the mounted Multiplex mapping of a vineyard are described in detail elsewhere (Diago et al., manuscript in preparation). They were not relevant for this study, where NBI maps were only used to choose the sampling sites that will allow a sufficiently large span of variable situations.

Chlorophyll Extraction. Leaf disks were collected immediately after the optical measurements. They were frozen in liquid nitrogen and stored at −80 °C until further processing. Disks were powdered in liquid nitrogen and extracted three times at room temperature with methanol (3 × 1.5 mL) containing calcium carbonate.[19] The three supernatants obtained by centrifugation (10000*g*, 5 min) were grouped, topped to 5 mL, and centrifuged again at 4100*g* for 5 min. The extinction coefficients for Chl in pure methanol of Porra et al.[38] were used to calculate the Chl concentration in the extracts.

Leaf Nitrogen Content. In the Leaf 2007 and Leaf 2009 data sets, individual leaf disks were freeze-dried, and after fine grinding, a 2−3 mg aliquot of the powder was analyzed by the Dumas method using isotope ratio mass spectrometry coupled with elemental analysis (EA−IRMS) using the analytical platform Metabolism-Metabolome of Labex Saclay Plant Science (France). This method measures the total N content, including nitrate. For the Plots 2009 data set, 120 disks were pooled together per plot and the aliquots were analyzed as described above. In the Subplots 2012 data set, nine whole leaves were pooled together per subplot sample after the optical measurements. Leaves were dried at 65 °C until a constant weight was obtained and then ground. Total leaf nitrogen content was determined in 50 mg aliquots for each subplot by the Kjeldahl method in a commercial laboratory (Laboratoire LCA, Bordeaux, France).

Statistical Analysis and Plotting. Data were treated, transformed, statistically analyzed, fitted, and plotted using a combination of software: Excel 2003 (Microsoft, Redmond, WA), Statistica 6 (StatSoft, Tulsa, OK), and Igor Pro 6.02 (WaveMetrics, Portland, OR). The accuracies of the fitted models and of the prediction of leaf N content from Chl and Flav contents and the NBI index were assessed by the coefficient of determination (R^2) and the root mean square error (RMSE):

$$\mathrm{RMSE} = [1/n \sum_{i=1}^{n} (\hat{y}_i - y_i)^2]^{1/2} \quad (1)$$

where $\hat{y}_i$ is the model-predicted value and y_i the measured value. "Major axis" regressions were used in all of the presented graphs, considering that the error is present in both plotted variables.[39]

Other descriptors listed in Tables 1 and 2 for the classification test are defined as usual,[40] using the true positive (TP), false positive (FP), false negative (FN), and true negative (TN) values present in the contingency table (Table 1) that compares the diagnostic test to the gold standard (here the wet chemistry N analysis):

$$\text{sensitivity} = \mathrm{TP}/(\mathrm{TP} + \mathrm{FN}) \quad (2)$$

$$\text{specificity} = \mathrm{TN}/(\mathrm{TN} + \mathrm{FP}) \quad (3)$$

$$\text{Youden index} = \text{sensitivity} + \text{specificity} - 1 \quad (4)$$

$$\text{accuracy index} = (\mathrm{TP} + \mathrm{TN})/(\mathrm{TP} + \mathrm{TN} + \mathrm{FP} + \mathrm{FN}) \quad (5)$$

The number of leaves needed to establish reliable sampling guidelines was calculated according to Dagnelie:[41]

$$\text{sample size} = k(\mathrm{CV})^2/\partial^2 \quad (6)$$

where CV is the coefficient of variation of the sample and ∂ the relative difference among plots that we want to detect. The parameter k is approximately 21 and 40 for acceptable errors of 10% and 5%, respectively.

Table 1. Example of a Contingency Table for the Calculation of the Youden Index[a]

	leaf nitrogen content		
	nitrogen + (N+)	nitrogen − (N−)	
optical index + (I+)	TP	FP	sum of I+
optical index − (I−)	FN	TN	sum of I−
	sum of N+	sum of N−	sample size (n)
	sensitivity	specificity	Youden index

	leaf nitrogen content		
	N+	N−	
I+	15	1	16
I−	1	10	11
	16	11	27
	0.94	0.91	0.85

[a]Data are for the chlorophyll index of the Plots 2009 data set (Figure 3): true positive (TP), false positive (FP), false negative (FN), and true negative (TN). For index definitions and calculation formulas, see the Materials and Methods.

Table 2. Statistics for the Calibration Models and Classification Test Indices for the Optical Indices[a]

optical index		Leaves 2007	Leaves 2009	Plots 2009	Subplots 2012
	N threshold (mg/g)	35	40	35	30
	sample size (n)	12	59	27	31
NBI	RMSE	2.5	3.7	1.7	1.8
	R^2	0.740	0.488	0.907	0.844
	sensitivity	0.71	0.79	0.94	0.77
	specificity	1	0.88	1	0.94
	Youden index	0.71	0.67*	0.94	0.71
	accuracy index	0.83	0.83	0.96	0.87
Chl	RMSE	2.2	4.5	2.0	2.6
	R^2	0.790	0.387	0.877	0.722
	sensitivity	0.71	0.74	0,94	0.85
	specificity	0.80	0.72	0.91	0.89
	Youden index	0.51	0.46***	0.85	0.74
	accuracy index	0.75	0.73	0.93	0.87
Flav	RMSE	4.1	7.6	2.3	3
	R^2	0.498	0.184	0.840	0.659
	sensitivity	0.71	0.44	0.94	0.77
	specificity	1	0.52	1	0.94
	Youden index	0.71	−0.04***	0.94	0.71
	accuracy index	0.83	0.48	0.96	0.87

[a]Samples were made of individual leaves for Leaves 2007 and Leaves 2009. Samples were averages of 60 leaves for Plots 2009 and averages of 9 leaves for Subplots 2012. RMSE is expressed in N units. All R^2 values were highly significant ($p < 0.001$). The t test was applied to verify the significance of the differences among the Youden indices of optical indices, both among data sets and among optical indices. The p values for the differences are indicated using asterisks: *, $p < 0.05$; ***, $p < 0.001$.

RESULTS AND DISCUSSION

Calibration of Optical Indices against Leaf N. Although the Dualex can be used on any crop or plant species, we decided to present here the specific case of grapevine, which is a crop and a ligneous species. We calibrated the Dualex against leaf nitrogen content as the "gold standard" by using natural variability of the nitrogen supply to grapevine due to soil heterogeneity, as opposed to controlled fertilization experi-

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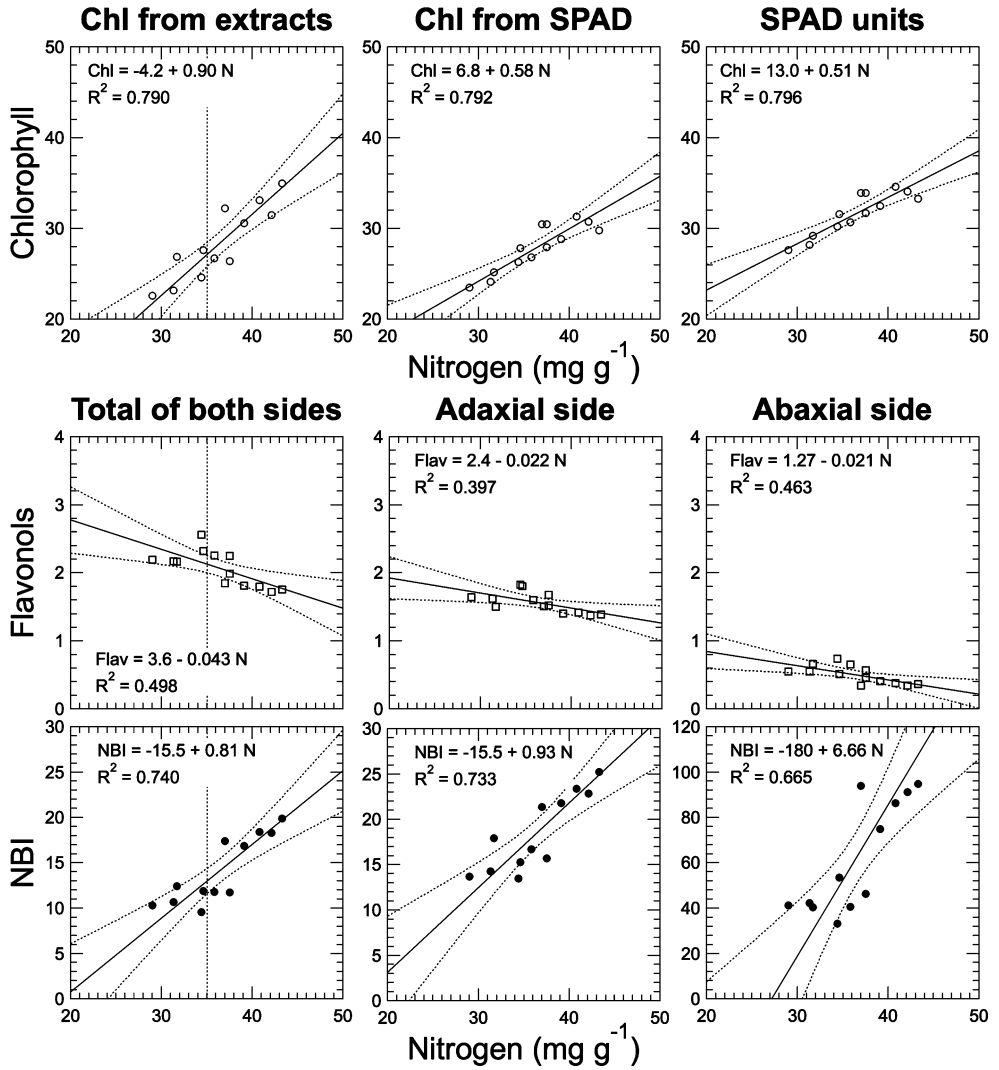

Figure 1. Dependence of optical indices on individual leaf nitrogen content for the Leaves 2007 data set. Each datum represents an individual leaf. The three top graphs are for Chl: total Chl content measured from extracts (left), Chl measured with the SPAD-502 (right), and Chl calculated from the SPAD-502 measurements using the consensus equation from Cerovic et al.[19] (center). Three Flav indices are presented in the middle row of the figure: Flav measured from the abaxial side of the leaf (right), Flav measured from the adaxial side of the leaf (center), and the sum of Flav of the two sides (left). All three NBI indices, total (left), adaxial (center), and abaxial (right), were calculated using the same transformed SPAD-502 measurements (center Chl graph). Regression models are indicated on the graphs by a full line, with dotted lines representing the 95% confidence interval bands. The vertical dotted line indicates the threshold limit used for the classification test presented in Table 2.

ments that usually yield a smaller number of data points and ambiguous results on ligneous perennials.[15] The responses of Chl, Flav, and NBI to N content in individual leaves are presented in Figures 1 and 2, and responses at the level of leaf populations, whole plots or subplots, are presented in Figures 3 and 4, respectively. All figures were plotted with the same N range for an easy visual comparison, although the different data sets did not have the same N average, span, or range. This is especially the case for Subplots 2012 (Figure 4) that was sampled two months later (end of July) than the other three data sets (end of May) (Figures 1−3). Linear regression models were sufficient and were the best in all cases. Determination coefficients (R^2) are indicated in the graphs and in Table 2. Table 2 also includes the corresponding RMSE. Using both criteria and for all three optical indices, Plots 2009 gave the best results with the smallest RMSE for the estimation of N content of 2.3, 2.0, and 1.7 mg g^{-1} for Flav, Chl, and NBI, respectively (Table 2). This is a consequence of the larger number of leaves averaged in the Plots 2009 data set. Data for the three cultivars were analyzed together. This showed the universality of the calibration, at least for these three cultivars. In all four data sets, Chl was better related to N than was Flav, with RMSE for Chl almost 2 times smaller for individual leaves. They were 2.2 vs 4.1 mg of N g^{-1} DW and 4.5 vs 7.6 mg of N g^{-1} DW, for Chl vs Flav, respectively (Table 2). The difference was much smaller in the case of plots and subplots where populations of leaves are sampled and averaged together, 2.0 vs 2.3 mg of N g^{-1} DW and 2.6 vs 3.0 mg of N g^{-1} DW, for Chl vs Flav (Table 2). Moreover, NBI was the best estimator of N content in all situations except for Leaves 2007, in which light-exposed and shaded leaves were mixed. There was a substantial decrease of NBI RMSE to 1.7 and 1.8 for plots and subplots and an increase in R^2 to 0.907 and 0.844 for Plots 2009 and Subplots 2012. Therefore, NBI, which is the ratio of Chl to Flav, improves the estimation of N even though it combines two estimators of inferior quality.

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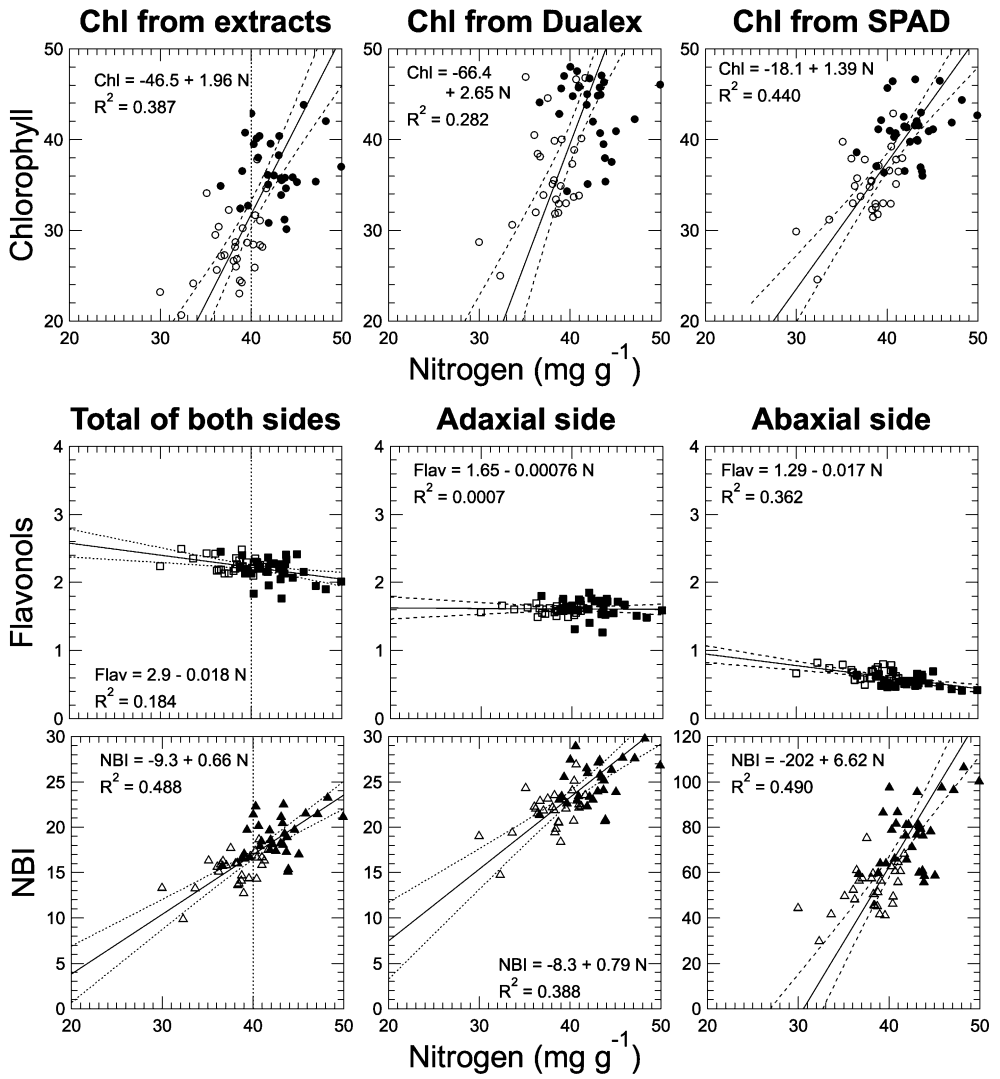

Figure 2. Dependence of optical indices on individual leaf nitrogen content for the Leaves 2009 data set. Each datum represents an individual leaf. Open symbols were used for Chardonnay and closed symbols for Pinot noir. The three top graphs are for Chl: total Chl content measured from extracts (left), Chl measured with the Dualex (center), and Chl calculated from the SPAD-502 measurements using the consensus equation from Cerovic et al.[19] (right). Three Flav indices are presented in the middle row of the figure: Flav measured from the abaxial side of the leaf (right), Flav measured from the adaxial side of the leaf (center), and the sum of Flav of the two sides (left). All three NBI indices, total (left), adaxial (center), and abaxial (right), were calculated using the same transformed SPAD-502 measurements (right Chl graph). Regression models are indicated on the graphs by a full line, with dotted lines representing the 95% confidence interval bands. The vertical dotted line indicates the threshold limit used for the classification test presented in Table 2.

The calibration at the population level, 60 leaves at the plot level (Figure 3) and 9 leaves at the subplot level (Figure 4), which is the agronomical pertinent situation, decreased the variability compared to individual leaf calibration (Figures 1 and 2). Plots 2009 and Subplots 2012 were two very different data sets. The former was obtained for Champagne on Pinot noir at flowering and the latter for Bordeaux on Merlot noir at bunch closure. The two month difference was the major factor governing the differences seen between the two.[16] Flavonol content in Subplots 2012 was greater because flavonols were accumulated with time and light exposure. Leaf N content was smaller because it was diluted with time and LMA increase.[6,42] This had a cumulated consequence on the NBI that also decreased. The data obtained here for Pinot noir (Figure 3) and Merlot noir (Figure 4) correspond to those obtained by Romero et al.[6] on Tempranillo. In both cases, the average N content decreased from 36 mg g^{-1} at the end of May to 28 mg g^{-1} at the end of July. Contrary to our expectations, a precisely controlled situation in which local Dualex sampling on leaf disks was compared to a chemically tested leaf N on the very same disk (Figures 1 and 2) did not produce better calibrations than the mixed-leaf N analysis (Figures 3 and 4). Pooled leaves from a single height without even counting the nodes (Figure 4) produced better results than single leaves (Figures 1 and 2).

Nitrogen Balance Index. The NBI correlates better with N content on a mass basis because it takes into account not only the surface-based variability in N investment in the photosynthetic machinery but also the variation in LMA. This advantageous relationship could be obtained by comparing surface-based Chl with surface-based N after its transformation with the LMA (surface-based N = mass-based N × LMA), but unfortunately, the estimation of LMA is destructive. In ligneous species light controls N investment in leaves to adjust photosynthesis to its light environment (photosynthetically

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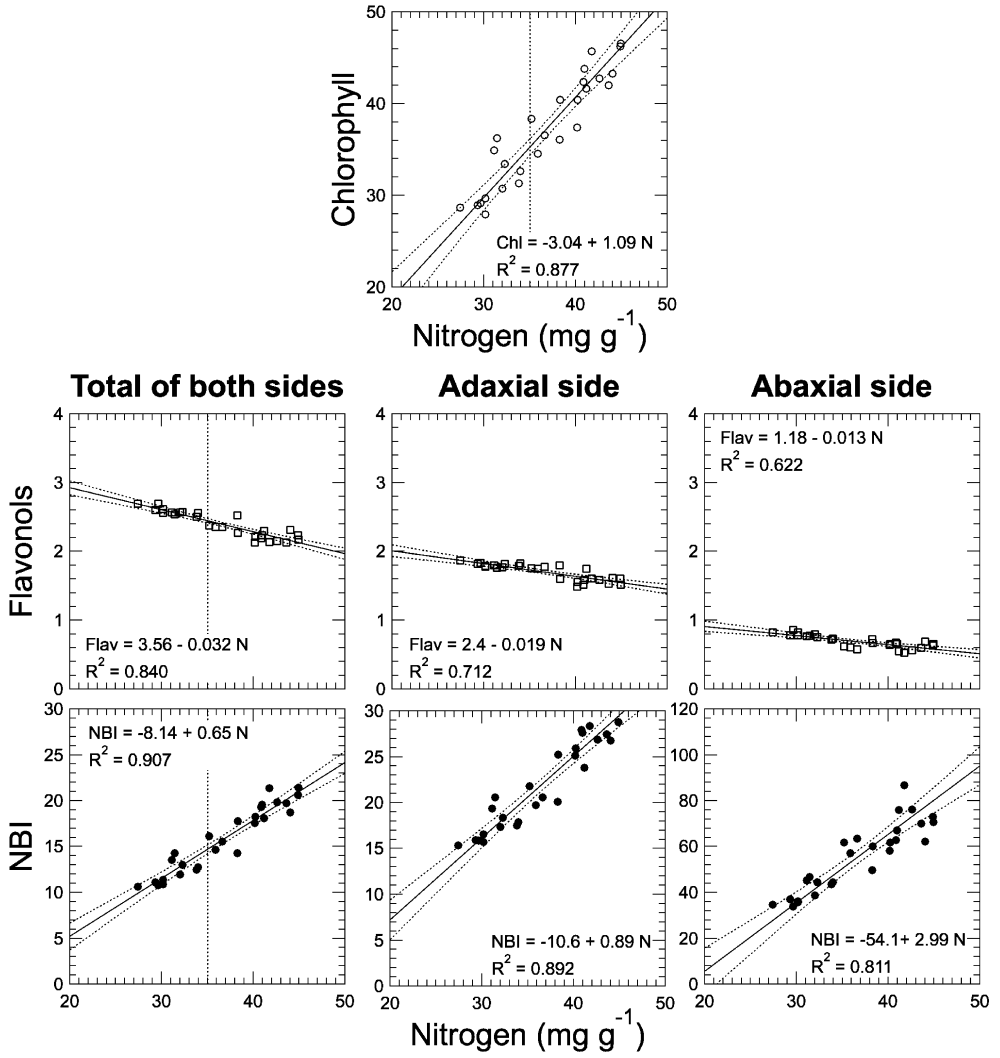

Figure 3. Dependence of optical indices on the mean nitrogen content for the Plots 2009 data set. Each datum represents a single plot characterized by the average of 60 leaves. The top graph is for Chl calculated from the SPAD-502 measurements using the consensus equation from Cerovic et al.[19] Three Flav indices are presented in the middle row of the figure: Flav measured from the abaxial side of the leaf (right), Flav measured from the adaxial side of the leaf (center), and the sum of Flav of the two sides (left). All three NBI indices, total (left), adaxial (center), and abaxial (right), were calculated using the same transformed SPAD-502 measurements (top Chl graph). Regression models are indicated on the graphs by a full line, with dotted lines representing the 95% confidence interval bands. The vertical dotted line indicates the threshold limit used for the classification test presented in Table 2.

active radiation (PAR) irradiance) by increasing LMA (mostly leaf thickness) without changing mass-based N.[43] This has been shown for grapevine,[34] for beech,[44] and for tropical trees.[45] This is why surface-based leaf Chl content obtained with leaf clips might not always correlate to leaf N. Surface-based Chl will reflect leaf N only when comparisons are made among leaves that developed under different N supplies but under the same light regime. Using Flav as a proxy for LMA[16,30] can compensate for different leaf light exposures. Therefore, the introduction of the NBI index will alleviate the frequent problem of surface-based Chl measurements compared to mass-based N (see, for example, the paper by Porro et al.[11]).

The abaxial Flav level was always lower than the adaxial level (Figures 1−4). Later in the season (Figure 4), both leaf sides had larger Flav contents, but the increase was more marked on the abaxial side. The single-sided Flav defines the single-sided NBI because Chl remains the same independent of the side from which it is measured (illustrated in Figure 4). Indeed, Chl in Figure 4 shows that there were no significant differences when it was measured from the adaxial or abaxial side, confirming that a single measurement is sufficient for leaf clips based on transmittance. In all four cases (Figures 1−4), the total NBI calculated using the sum of Flav of both leaf sides was better correlated to N (higher R^2) than single-sided NBI. In addition, it can be seen that sometimes single-sided NBI (adaxial NBI in Figures 1 and 3, abaxial NBI in Figure 2) gives estimations as good as the total NBI. Still, the absolute values of the total NBI are more stable. Single-sided measurements are important for the future use of noncontact fluorescence sensors such as the Multiplex that have a different response depending on the side or measurement[10] (Diago et al., manuscript in preparation), but this is outside the scope of the present paper.

Classification Test. For arable crops that have a quadratic response of yield to N supply, with saturation often above a threshold, the Cate−Nelson classification[46] can be used to separate the responding from the nonresponding regions in the yield-to-N supply curves. Here we have a linear relationship between optical indices and leaf N, so the Cate−Nelson

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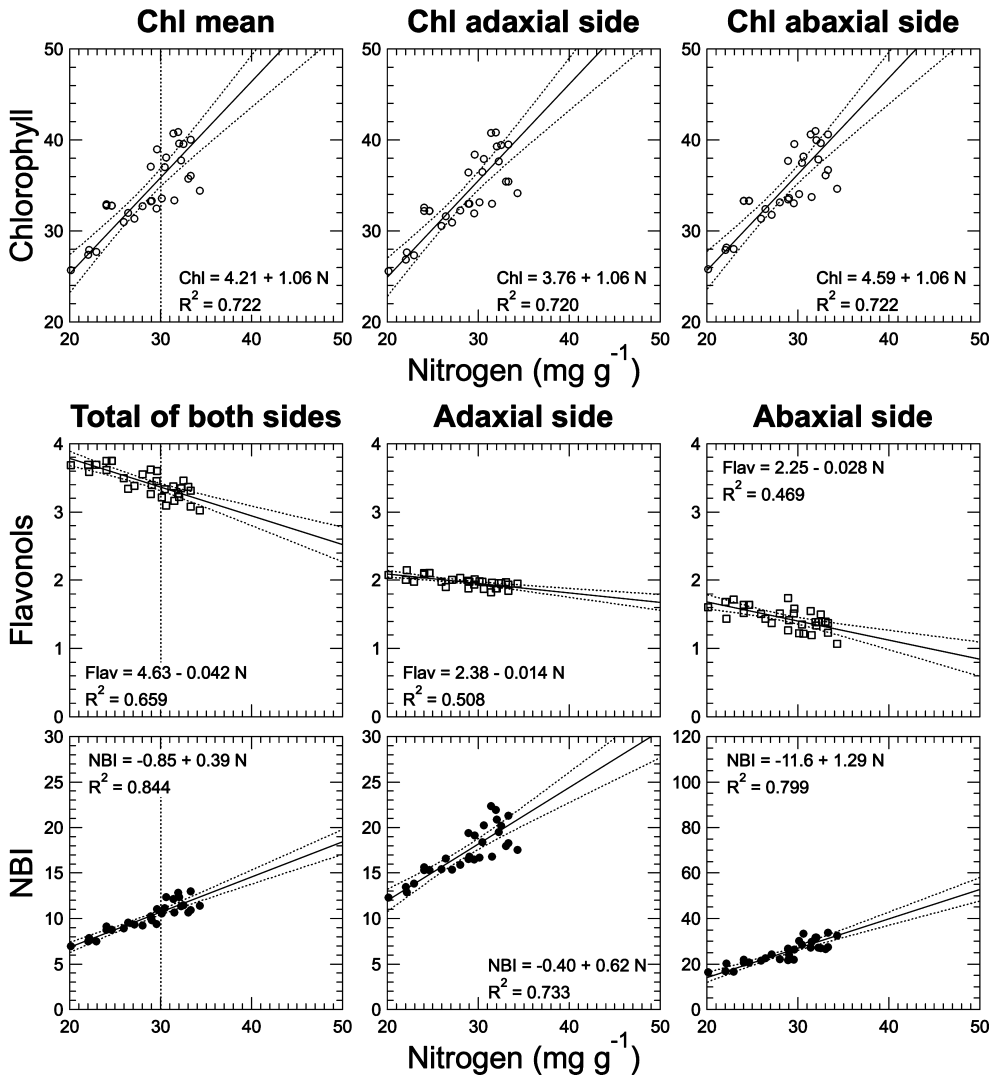

Figure 4. Dependence of optical indices on the mean nitrogen content of leaves for the Subplots 2012 data set. Each datum represents a subplot characterized by the average of nine leaves present on three adjacent vines. The three top graphs are for Chl measured with the Dualex 4 from the abaxial side of the leaf (right) and from the adaxial side of the leaf (center) and the mean Dualex Chl of the two leaf sides. Three Flav indices are presented in the middle row of the figure: Flav measured from the abaxial side of the leaf (right), Flav measured from the adaxial side of the leaf (center), and the sum of Flav of the two sides (left). The three NBI indices, total (left), adaxial (center), and abaxial (right), were calculated using the mean Dualex Chl and the adaxial and the abaxial Chl, respectively. Regression models are indicated on the graphs by a full line, with dotted lines representing the 95% confidence interval bands. The vertical dotted line indicates the threshold limit used for the classification test presented in Table 2.

statistical procedure was not well adapted. However, for practical purposes optical indices are usually used to diagnose deficiency and classify plots to those needing fertilization or not, thanks to their relationship to leaf N. This is why the presented continuous values (Figures 1−4) can also be analyzed on the basis of a cutoff value for sufficient leaf N. An evaluation of the test for this binary classification can be done by calculating the Youden index (Youden's *J* statistics, informedness)[40] (Table 1). This index is a commonly used measure for overall diagnostic effectiveness of a method. It is especially useful because it combines sensitivity and specificity, which are both prevalence-independent statistics. Leaf N content is considered here as the gold standard for N sufficiency. Generally, levels of N in leaf blades below 20 mg g^{-1} between flowering and véraison indicate deficiency[47] (see Table S1, Supporting Information). For the four different data sets, we had to apply different threshold values to have an acceptable prevalence (proportion of positive values) for the contingency table analysis. The threshold values (cutoffs) were chosen to have a prevalence of around 50%. In addition, they corresponded to the real situation generally recorded in the field (cf. Table S1). The individual leaf data sets (Figures 1 and 2) and the plot data set (Figure 3) for Champagne cultivars were obviously on the high N side; therefore, 35 and 40 mg g^{-1} thresholds were tested (Table 2). For the Leaves 2009 data set, we used a higher threshold (40 mg g^{-1}) because for the 35 mg g^{-1} threshold the prevalence was too large to be acceptable (0.89).[40] According to Youden,[40] the sample size was sufficient for comparisons except for the Leaves 2007 data set, which only had 12 leaves. No significant differences among indices (NBI vs Chl vs Flav) or among data sets (type of data sets) were found, apart from the Leaves 2009 data set, for which the Youden index for Flav was much lower than that for Chl and NBI (Table 2). In plant disease research, as in medicine, one would

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favor tests that minimize false negatives, that is, tests that maximize sensitivity. On the contrary, farmers' adversity toward loss of yield would prefer an indicator that minimizes false positives, that is, which maximizes specificity. They prefer to fertilize even if it is not needed, rather than to lose some yield. In viticulture at the lower threshold (N-deficient vines), a viticulturist would behave like any other farmer, but at the higher threshold (N overfertilization), he should behave like a physician: he would favor an indicator that avoids false negatives because overly vigorous vines are more prone to diseases and produce grapes of lower technological quality.[4]

The Leaf 2009 data set, which had the largest dispersion of experimental points, is characteristic of the advantage of NBI over the use of Chl and Flav indices alone. Using a poor Flav index that has low sensitivity and specificity (Youden's $J = 0$) to divide a modest Chl index that has a modest sensitivity and specificity (Youden's $J = 0.46$) produced an NBI index that has a fair sensitivity (0.79) and a high specificity (0.88). The Youden index for NBI increased to 0.67, and accuracy reached 0.83. This is an outstanding achievement for an index that has an RMSE of 3.7 mg g^{-1}, which is 2 times larger than the plot (1.7) and subplot (1.8) RMSEs (Table 2). We can conclude that both the Youden index and the accuracy of the leaf-clip method are very good for N nutrition estimation in grapevine. There were two major reasons for this success. First, Dualex had a more linear response than SPAD-502 thanks to the choice of a less absorbed sensing wavelength.[19,48] However, the use of the consensus equation[19,48] for the transformation of SPAD-502 units also helped to obtain quantitative relationships. Second, grapevine is a crop usually fertilized with parsimony; therefore, leaves rarely attain the very high levels of N and Chl found in overfertilized grain crops.[49] Even then, it is expected that epidermal leaf Flav will continue to respond to increased N when Chl stops responding.[10,21,22]

Choice of the Representative Leaf and Growth Stage. Reliable estimation of N content will be dependent on several factors: the most representative leaf, the number of leaves to represent the management unit, the best plant, and the leaf phenological stage.[3,8] In a preliminary experiment, we measured all leaves of all primary shoots of a Merlot noir vine on three dates in July 2014. The within-plant coefficients of variation for Chl, Flav, and NBI were 25%, 12%, and 31%, respectively. Therefore, to discriminate a 10% difference in Chl content with an acceptable error of 10%, a minimum of 131 leaves would be needed to reliably sample a plot (250 leaves for an error of 5%)[41] (see the Materials and Methods). Fortunately, the variation among the leaves at the same node number is much smaller than that, ranging from 6.6% to 14%. By fixing the node position, the needed number of sampled leaves decreases to a minimum of 9 and a maximum of 45 for an acceptable error of 10% (41−78 leaves for an error of 5%). The intraleaf variability can be even smaller. Its coefficient of variation was often 4%, 6%, and 8.5% for Chl, Flav, and NBI, respectively. Therefore, with a measurement on a single well-defined position on a leaf, one could concentrate the sampling effort to different vines because it is advantageous to sample leaves from different plants in a sufficient number to obtain a smaller variance in N estimation.[8]

The leaf opposite the first cluster on node no. 4 counted from the base of the shoot is often used for N analysis.[11] At flowering this leaf corresponds to a just-matured leaf, but at later phenological stages it is not the best choice because it becomes very old. Therefore, attempts were made to replace it by the next leaf just above it, i.e., the leaf opposite the second cluster, or even the leaf four nodes above it.[5,50] It is obvious that a metabolically active younger leaf would be a better choice.[51] If 4−5 nodes are counted above the clusters, as preconized in Italy[50] for later growth stages, it would have forced us to select a leaf on node nos. 8−9 from the base. For a 13−14-leaf shoot, it will actually correspond to leaf no. 5 from the tip. Often used in phytopathology research,[52] leaf no. 5 from the tip is representative of the leaf that has just attained maturity. From an expanding carbon sink leaf, it becomes a carbon source leaf; therefore, it is more sensitive to deficiencies. The universally accepted node and the leaf position for N sampling that we recommend here is around the ninth node from the shoot base for a 14-node fully developed shoot. This leaf will be in the upper half of the vertical shoot positioning (VSP) trained vines, above the second wire in a three-wire trellis system, or just below the second wire in a two-wire trellis system. This would also be the best position for the noncontact on-the-go sensing with optical sensors such as the Multiplex[32] or the Crop Circle.[12]

In addition to postharvest autumnal fertilization, flowering is the best phenological stage for in-season fertilization[34] because at this stage it is still possible to correct for N deficiency by in-season amendments if needed. Véraison, the stage at which most diagnoses are often performed (Table S1, Supporting Information), is too late for efficient in-season amendment.[51] It can only be useful as information for viticultural practice decisions concerning next year's crop, such as the severity of pruning and potential interrow cropping. Therefore, performing leaf N diagnosis somewhere between flowering and bunch closure (BBCH 65−75) would be recommended.

Leaf Nitrogen Norms. For various plant species and independently of the growth stage, N contents in mature leaf tissue of less than 25, between 25 and 45, and above 60 (mg of N g^{-1} DW) are usually considered as deficient, sufficient, and excessive, respectively.[3] In grapevine, excess N can be even more damaging than deficiency because vines would be more subject to diseases and insect infestations.[53] Overfertilization usually produces grapes of lower quality.[4] Overfertilized plants are also prone to flowering abortion and a lack of fruit set.[3,54] Therefore, for grapevine these N thresholds were defined for individual countries or even wine-growing regions[55,56] (cf. Table S1, Supporting Information). For example, for Washington, the critical range proposed is 25−35 mg of N g^{-1} DW at bloom and 22.5−32.5 mg of N g^{-1} DW at véraison.[57] For Bordeaux, leaf N contents at véraison of 22.0, 26.2, and 34.1 mg of N g^{-1} DW were considered limited, mean, and excessive by Hilbert et al.[35] (cf. Table S1).

Calibrations performed in the present work allow us to propose the corresponding values of Chl and NBI for the consensus thresholds drawn from the literature at flowering and bunch closure (Table 3). On the basis of the literature survey, we have chosen 30 and 40 mg of N g^{-1} DW for the lower and upper thresholds at flowering and 22 and 32 mg of N g^{-1} DW at bunch closure. The corresponding Chl values are 30 and 40 μg cm^{-2} for flowering and 29 and 37 μg cm^{-2} for bunch closure. The corresponding NBI values are 11 and 18 for flowering and 8 and 11 for bunch closure.

When proposing the use of absolute thresholds, it is important to estimate the associated uncertainty. Several factors should be taken into account: leaf position (10% error), phenological phase at time of diagnosis (5%), inherent precision (or lack of precision) of the optical diagnostic

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Table 3. Tentative Grapevine Leaf Content Norm Thresholds for Nitrogen (mg g^{-1}), Dualex Chlorophyll (μg cm^{-2}), and Dualex NBI Index at the Flowering and Bunch Closure Growth Stages

	deficient	sufficient (optimal)	excessive (overfertilized)
		Flowering	
nitrogen	<30	30−40	>40
chlorophyll	<30	30−40	>40
NBI index	<11	11−18	>18
		Bunch Closure	
nitrogen	<22	22−32	>32
chlorophyll	<29	29−37	>37
NBI index	<8	8−11	>11

method (10%), and the precision of the gold standard (5%). The differences in estimation of N content by the reference methods can be substantial because they do not analyze the same type of N. The Dumas combustion method includes all types of N (NH_4, NO_3, and protein and heterocyclic N). The N_2 produced is determined by mass spectroscopy or by thermal conductivity detection with reproducibility not better than 5%. The Kjeldahl digestion method, until now the industry standard, yields lower results than the Dumas method because it neglects heterocyclic N. It can be performed with or without NO_3 and NO_2 recovery, which changes the results among laboratories.[8] Therefore, if we want to use an absolute threshold calibrated against the gold standard, the overall uncertainty of optical estimation including all factors will not be smaller than 16%. The latter value was calculated as the square root of the sum of squares of individual errors assuming that individual errors were independent and uncorrelated. This is larger than the year-to-year variability of ±10% found by Failla et al.,[56] corresponding to an average of 20 mg of N g^{-1} DW ± 2 mg g^{-1}. The regional variability due to soil and climate and the year-to-year variability are what we are looking for, so they were not included in this calculation. In summary, with the best protocol, a plot can be declared N-deficient with certainty if it has an average Chl content lower than 25 μg cm^{-2} at flowering. It can be considered overfertilized if it has a Chl above 47 μg cm^{-2}.

Proposed Protocol. All of the above work incites us to propose a stringent sampling protocol comprising double-sided Dualex measurements on 40 light-exposed primary leaves positioned at the same canopy height, just above the first trellis wire in a two-wire trellis system, which corresponds to the middle of the canopy (i.e., node no. 9). The optimal procedure would be to divide the plot to be diagnosed into three equal parts and to walk through the two interrows that separate these parts. One should then sample (measure with the Dualex) one leaf from the three shoots of a vine, repeating this on five vines from each side of the interrow, left and right, and for the two interrows. This would yield measurements from 60 leaves all together (1 leaf × 3 shoots × 5 vines × 2 row sides × 2 interrows). A single measurement per leaf at the intervein position (Figure S1, Supporting Information) is sufficient. The representative average for the plot for Chl and for NBI (Chl divided by the sum of Flav of the two leaf sides) can be compared to the threshold criteria presented in Table 3. Alternatively, for smaller plots or for visually homogeneous plots, the sampling can be decreased to 36 leaves (1 leaf × 3 shoots × 3 vines × 2 row sides × 2 interrows). Thanks to the knowledge of leaf N dilution during the season,[5,6] a lookup table could also be constructed to correct for seasonal effects when diagnostics cannot be made at the optimal postflowering growth stage (the BBCH 65−75 window). To sum up, we propose to sample 36−60 leaves at flowering at a fixed node position, preferably node no. 9, or at the same height for VSP.

Limitations of Optical Nitrogen Estimation. Water availability influences nitrogen uptake[58] and therefore would influence leaf N content. One should make a distinction between the effect of water availability on nutrient analysis[59] and genuine leaf N content physiological dependence on rainfall.[58] In the first case, a water deficit simply precludes the use of petiole nitrate tests, and leaf N is also affected. In the second case, water availability, rainfall, or irrigation during canopy development interacts with nitrogen availability to optimize simultaneously both nitrogen and water use efficiency by adjusting the total leaf area, N per leaf area, and LMA. This represents the potential limit for the use of absolute values of NBI because Flav is also influenced by light and water stress.[60] As an alternative approach to NBI, Flav can be used to verify that sampled leaves belong to the same sun-exposed category that has the same LMA. In that case, Chl alone could be used as a proxy for N content even when expressed in surface-based units. Then the Chl index would perform sufficiently well and can be used as an absolute value. Knowing that only thoroughly trained and experienced technicians should be responsible for collecting plant tissue samples[8] and that very often this is not the case, this type of objective postacquisition filtering of optically acquired data could be very useful.

Other leaf-clip chlorophyll meters such as the SPAD-502 can also be used for N estimation.[11−15] They would best be used after an adequate transformation into leaf Chl content (cf. the Material and Methods). Still, the SPAD-502 leaf clip has an effective access length of only 1.3 cm because it was originally designed for rice leaves. This is why in the present study we performed the measurement with the SPAD-502 on the disks once they had been cut from the leaf. This can be a real problem for rapid in-field measurements if one wants to avoid leaf edges. To scarify one leaf among the two thousand leaves present on a vine by cutting it might not seem prohibitive, but it will greatly slow the diagnostic speed. The Dualex that was designed for grapevine leaves has an access length of 8 cm to attain the middle of even a 16 cm wide leaf.

The "nitrogen sufficiency index" approach[1] is another potential way to alleviate most of the problems of the use of absolute threshold values for the indices because it is related to a standard condition for the same index. It could mitigate the above-mentioned concerns about both the absolute NBI values and the environmental and phenological influences on N estimation, such as the variable growth rate and leaf age at the time of measurement. Unfortunately, the nitrogen sufficiency index cannot be applied to a ligneous species such as grapevine on a year-to-year basis due to a lack of overfertilized reference and to the latency in N deficiency expression. However, a new approach to explore within the framework of site-specific crop management could be to use a virtual-reference concept,[61] i.e., just the comparison of subplots or homogeneous zones to the one having the largest NBI value. Indeed, for spatial heterogeneity analysis and zoning, both Chl and NBI measurements are valuable, especially with tractor-mounted sensors such as the Multiplex.

Conclusion and Prospects. Technology-based optical sensing is the equivalent of visual estimation. Still, it is more objective and can be automated and georeferenced. It is well

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adapted for site-specific crop management in replacement of plant tissue testing. In this paper we presented quantitative optically measured norms for grapevine N nutrition based on extensive studies of Chl and Flav using a new optical leaf clip. The knowledge of the growth stage and the sampling of leaves at a defined node are crucial for a reliable estimation of grapevine N status. The presented grapevine optically measured leaf N sufficiency level thresholds should be refined for different phenological stages, from flowering to véraison, by accumulation of data on different sites. The universality of Chl and NBI indices should be verified for different cultivars. Their robustness against year-to-year variability should also be verified by multiyear surveys on the same sites. Finally, for practical applications of these optical approaches in precision viticulture, indices obtained by mounted optical proximal sensors such as the Multiplex need to be calibrated and validated against the Dualex indices presented in this paper. Grapevine is just an example of the use of optical sensors for the applications of leaf Chl and NBI for crop N management. In viticulture, it is important to estimate vine nitrogen availability for three reasons. First, N affects yield, as in other crops. Second, it influences the grape fermentation potential.[35] Third, it defines the must quality via phenolic and technological grape maturity. The type of analysis presented in this paper can and should be repeated on other crops. Indeed, some data are already available for muskmelon.[28]

■ ASSOCIATED CONTENT

Supporting Information

Table S1 listing literature data for the total nitrogen content (% DW) in leaf blades at four phenological stages, Figure S1 showing the preferred positions for optical measurements on the leaf, and Figure S2 showing the NBI map of the plot at Chateau Gazin with sampling points indicated by green circles for data set Subplots 2012. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +33169157224. Fax: +33169157353. E-mail: zoran.cerovic@u-psud.fr.

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Notes

The authors declare the following competing financial interest(s): Z.G.C. declares a double link to the FORCE-A company: as one of the co-authors of the Dualex patent that the company exploits and as a part-time consultant to the company. Other authors declare no competing financial interests.

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■ ABBREVIATIONS USED

N, nitrogen; NBI, nitrogen balance index; Chl, chlorophyll; LMA, leaf mass per area; Flav, flavonol; RMSE, root mean squared error; UV, ultraviolet; DW, dry weight

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