Calibration of non-invasive fluorescence-based sensors for the manual and on-the-go assessment of grapevine vegetative status in the field

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Abstract

Background and Aims: Optical sensors can accomplish frequent and spatially widespread non-destructive monitoring of plant nutrient status. The main goal was to calibrate a fluorescence sensor, used both manually (MX_H) and on-the-go (MX_M), for the assessment of the spatial variability in the vineyard of the concentration of chlorophyll, flavonol and nitrogen in grapevine leaves, against that of a leaf-clip type optical sensor (DX4).

Methods and Results: Measurements were taken in a commercial vineyard on the adaxial and abaxial sides of leaves of nine *Vitis vinifera* L. cultivars, manually with the DX4 and MX_H, and with the MX_M mounted on an all-terrain vehicle. A significant correlation was obtained for the chlorophyll and nitrogen indices of MX_H and DX4 ($R^2 > 0.90$) and of MX_M and DX4 ($R^2 > 0.74$), and the calibration equations were defined. A similar spatial distribution was achieved for the chlorophyll, flavonol and nitrogen indices of the leaves.

Conclusions: The capability of the fluorescence sensor, used manually and on-the-go, for characterising the nutritional status of grapevines was demonstrated.

Significance of the Study: This work reports the first calibration of the hand-held and on-the-go fluorescence sensor to assess key nutritional parameters of grapevines. The applicability of this sensor on-the-go to characterise the spatial variability of the vegetative status of a vineyard for the delineation of homogeneous management zones was proved.

Keywords*: grapevine, nitrogen, optical sensor, precision viticulture, proximal sensing*

Introduction

Vineyards have been demonstrated to be spatially variable. Within a vineyard, changes in soil type or depth, slope and exposure may occur. Each individual factor and the interaction among them influence grapevine development, leading to differences in vine growth, yield or grape composition (van Leeuwen 2010). The knowledge and study of the spatial variability of the several features of a vineyard allow a differentiated, optimised management, which is known as precision viticulture. For this purpose, the collection and use of large amounts of data related to the plant's physiological status, yield and grape composition are needed (Proffitt 2006).

Chlorophyll (Chl), flavonols (Flav) and nitrogen (N) are key physiological constituents of grapevines. Chlorophyll is the pigment responsible for photosynthesis and increases until grapevine leaves are fully expanded and starts to decrease afterwards, as soon as it attains its maximal value (Kriedemann et al. 1970). Flavonols comprise a class within the flavonoids, a secondary metabolite group of compounds sharing a three-ring phenolic structure. Flavonols in plants display a wide range of physiological functions, involving microbial interactions (Koes et al. 1994) and free radical scavenging (Markham et al. 1998), but their most prevalent role appears to be as UV screening agents (Flint et al. 1985, Smith and Markham 1998). Flavonol biosynthesis is upregulated not only because of UV radiation but also in response to other biotic and abiotic stresses, such as N/phosphorus depletion (Lillo et al. 2008), low temperature (Olsen et al. 2009) and salinity/drought stress (Tattini et al. 2004, Agati et al. 2011a). Leaf Chl and Flav concentration on a surface basis depends on leaf age and the amount of light radiation received during their development. Both increase with leaf expansion and light exposure until veraison, while afterwards, leaf Chl usually decreases (Louis et al. 2009) while Flav remain unvaryingly high (Downey et al. 2003). Nitrogen is considered to be one of the most important factors for biomass production (Lemaire et al. 2008, Agati et al. 2013a) and grapevine metabolism, as it is crucial for vine development and fruit yield (Guilpart et al. 2014). Therefore, the assessment of the vineyard Chl and N status is necessary and helpful to delineate strategies for fertilisation and canopy management intended to improve the grapevines' balance and fruit composition. In grapevines, excessive N can sometimes be even more damaging than N deficiency because vines would be more prone to disease and insect infestations (Dordas 2009). Overfertilisation usually produces grapes of poorer composition (Keller 2010), and plants are more susceptible to abortion of flowers and reduced fruitset (Vasconcelos et al. 2009).

Cartelat et al. (2005) have shown that both flavonol and Chl concentration is important for the assessment of the N status of the plant. This ratio is known as the N balance index (NBI = Chl/Flav), and its relationship with the N status has also been reported by several authors for other species (Tremblay et al. 2012). Chlorophyll concentration increases, whereas that of flavonol decreases with increased N application, so that the

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NBI increases with N fertilisation. Therefore, in the framework of precision farming, the epidermal concentration of flavonols and leaf Chl is useful for N management (Tremblay et al. 2012) as it allows the NBI to be calculated.

Leaf Chl, flavonol and N status are usually analysed with destructive wet chemistry methods. Compared with the latter, optical methods provide much faster assessment and are the only ones allowing practical whole plot analysis. Optical methods are based on leaf transmittance, reflectance or fluorescence, and they can be used as proximal sensors. Proximal sensing, which includes all detecting technologies that gather information from an object when the distance between the sensor and the object is less than, or comparable with, some of the dimensions of the sensor, have emerged as an alternative to remote sensing in viticulture. Proximal sensing provides a successful solution to most of the drawbacks, such as large proportion of background noise in the images, limited temporal flexibility and elevated cost of aerial monitoring, of remote sensing in vertically trellised vineyards worldwide. Among the wide variety of technologies used in proximal sensing, Chl fluorescence has been introduced in viticulture for the monitoring of anthocyanin accumulation, the assessment of vine vigour and the control of diseases in plants (Agati et al. 2008, Bellow et al. 2012, Latouche et al. 2013). It is possible to obtain estimates of anthocyanin and Flav by using the non-destructive Chl fluorescence excitation screening method: the higher the anthocyanin or Flav concentration in the berry skin or leaf, the lower the Chl fluorescence signal.

Proximal sensors can be either hand-held or mounted onto a machine, allowing both the acquisition of data in a non-destructive way (Tisseyre 2013). The spatial resolution of the data recorded, however—that is the number of measurements per unit plot surface—differs between the manual and the on-the-go operation. While the hand-held sensors are carried by an operator, who takes the measurements, the on-the-go devices are mounted onto a motorised vehicle [i.e. tractor and all-terrain vehicle (ATV)] and measure automatically according to a triggering protocol design. Therefore, the spatial resolution increases with the on-the-go sensors, and a large amount of data can be recorded in less time (Tisseyre 2013). Furthermore, when a global positioning system is used, the data points obtained can be georeferenced and interpolated to generate a comprehensive map of the crop condition (Tremblay et al. 2012). Tractor-based mapping would be extremely valuable, as tractors frequently move along the rows to undertake many vineyard operations. Therefore, by mounting sensors on tractors, information could be gathered with no time-cost and at different stages of vine development (Taylor et al. 2005).

The main goal of the present study was to calibrate against a leaf-clip optical sensor, used as calibrated reference, and to evaluate the performance of a portable non-destructive fluorescence sensor used both manually and on-the-go (on a motorised platform) for the assessment of the spatial variability and mapping of the concentration of Chl, Flav and N in grapevine leaves within a vineyard.

Materials and methods

Site description

The study was undertaken in 2012 during the last week of September and first week of October at a 1.43 ha commercial vineyard owned by the nursery Vitis Navarra located in Vergalijo (Latitude 42°27′45.96″, Longitude 1°48′13.42″, Altitude 325 m), Navarra, Spain. The vineyard was planted with nine red international cultivars: Cabernet Sauvignon, Carmenere, Caladoc, Grenache, Marselan, Maturana Tinta, Pinot Noir, Tempranillo and Syrah. Grapevines were trained to a vertically shoot-positioned trellis system, with north–south row orientation at 2 × 1 m inter-row and intra-row distances. Grapevines were planted on Richter 110, with the exception of Tempranillo vines, which were planted on rootstock 3309. Irrigation was routinely and uniformly applied across the season for all cultivars. The choice of a vineyard with several genotypes was made to increase the variability for the development of the calibration models and hence to develop a more robust model.

Fluorescence sensors and indices

The vineyard was monitored with three proximal sensors based on Chl fluorescence: the Multiplex, which was used manually [hand-held Multiplex (MX_H)] and on-the-go [Multiplex On-The-Go (MX_M)] and the leaf-clip fluorescence sensor, Dualex4 (DX4), which served as the reference device.

Leaf-clip fluorescence sensor

Dualex4 (FORCE-A, Orsay, France), DX4 hereafter, is a leaf-clip sensor with a measuring surface of 6 mm diametre, which measures leaf epidermal flavonols by the Chl fluorescence screening method (Goulas et al. 2004) and the Chl leaf concentration by differential transmittance (Cerovic et al. 2012). It provides three fluorescence indices: the chlorophyll optical index (CHL) (Equation 1) for the leaf Chl concentration, displayed in Chl units (Cerovic et al. 2012); the flavonol optical index (FLAV) (Equation 2.) for the epidermal Flav concentration in absorbance units; and the NBI index (Equation 3), as the ratio of Chl to Flav (Cartelat et al. 2005), which refers to the leaf N concentration (Cerovic et al. 2012).

$$\mathrm{CHL} = (T_{850} - T_{710})/T_{710} \quad (1)$$

$$\mathrm{FLAV} = \log(\mathrm{FRF_R}/\mathrm{FRF_U V}) \quad (2)$$

$$\mathrm{NBI_T} = [(\mathrm{CHL_{AD}} + \mathrm{CHL_{AB}})/2]/[\mathrm{FLAV_{AD}} + \mathrm{FLAV_{AB}}] \quad (3)$$

where T_{850} and T_{710} are the leaf transmittance at 850 and 710 nm, respectively; FRF is the far-red Chl fluorescence emission (>710 nm) excited by red (_R, 650 nm) or UV (_UV, 375 nm) light; and the subscripts AD and AB refer to the adaxial and abaxial sides of the leaf, respectively.

Instrument calibrations

The DX4 CHL and FLAV indices have been validated in a previous work by Cerovic et al. (2012) against Chl extracts and Dualex3 FLAV index, respectively, and the robustness of these calibrations of Dualex4 for the assessment of Chl and Flav in grapevine leaves is described. In that study, the reproducibility and accuracy of the calibration were provided, as well as model statistics such as residual sum of squares (RSS), root mean square error (RMSE), bias (BIAS) and standard error of prediction corrected (SEPC). The method and technology for the measurement of Chl and Flav were not changed between the different Dualex versions (DX4, the one used in the present study, and previous one, Dualex 3). The same light-emitting-diode sources and filters (therefore wavelengths) are used. The DX4 NBI index has been previously validated by Cartelat et al. (2005) to assess the N status of wheat and recently by Cerovic et al. (2015) against the N concentration in grapevine leaves over a period of 5 years. The work of

Cerovic et al. (2015) demonstrates the robustness of the predictive capability of the NBI index against the N concentration determined by wet chemistry, and values for sensitivity, accuracy and RMSE are provided. In that same work (Cerovic et al. 2015), calibration of DX4 against Chl extracts was also provided and the same model statistics as for N were shown.

Hand-held fluorescence sensor

The Multiplex (FORCE-A), MX_H hereafter, is a hand-held, multi-parametric fluorescence sensor based on light-emitting-diode excitation and filtered-photodiode detection that are designed to work in the field under daylight on leaves, fruits and vegetables (Ben Ghozlen et al. 2010). The sensor illuminates a surface of 8 cm diameter at a 10 cm distance from the source. This device provides 12 signals and several signal ratios, among them the indices that are the object of the present study: SFR (Equations 4,5), FLAV (Equation 6) and NBI (Equations 7–9), which are defined as

$$SFR\_R = \frac{FRF\_R}{RFR} \tag{4}$$

$$SFR\_G = \frac{FRF\_G}{RF\_G} \tag{5}$$

$$FLAV = \log\left(\frac{FRF\_R}{FRF\_UV}\right) \tag{6}$$

$$NBI\_R = \frac{FRF\_UV}{RF\_R} \tag{7}$$

$$NBI\_G = \frac{FRF\_UV}{RF\_G} \tag{8}$$

The SFR index is linked to the Chl concentration of leaves. It is a simple fluorescence ratio (SFR) of far-red Chl emission (FRF, 735 nm) divided by red Chl emission (FR, 685 nm) under red (FRF_R and FR_R, respectively) (Equation 4) or green excitation (FRF_G and RF_G, respectively) (Equation 5). Because of the overlap of the Chl absorption and emission spectrum, re-absorption occurs at shorter wavelengths (RF) but not at longer wavelengths (FRF) (Gitelson et al. 1999, Pedrós et al. 2010). Therefore, SFR increases with increasing sample Chl concentration.

The FLAV index (Equation 6) compares the Chl fluorescence intensity emitted as far-red fluorescence under ultraviolet (FRF_UV) and red excitation (FRF_R), which represents a differential absorption measurement (in accordance with the Beer–Lambert law) that is proportional to the Flav concentration of the epidermis (Ounis et al. 2001, Agati et al. 2011b).

The NBI displayed in Equations 7 and 8 is related to the N status of the plant and proportional to the Chl-to-Flav ratio proposed by Cartelat et al. (2005) but simplified. It utilises only two signals as the ratio of FRF_UV and RF_R in NBI_R, or green excitation (RF_G) for NBI_G.

Besides the NBI_R and NBI_G given by Equations 7 and 8, respectively, we calculated also the NBI index (NBI_C) separately for the adaxial and the abaxial leaf sides, as well as for the whole leaf, based on the ratio between the SFR and FLAV indices of the MX_H. The NBI_T index of Equation 9 is the calculated hand-held Multiplex index that corresponds to the one obtained with the DX4. It takes into account the total Chl concentration of the leaf (numerator) and the sum of the epidermal Flav of the abaxial and adaxial sides of the leaf (denominator).

$$NBI_T = \frac{Chl}{Flav} = \frac{SFR_{AD} + SFR_{AB}}{FLAV_{AD} + FLAV_{AB}} \tag{9}$$

Equations 10 and 11 show the formulae for the computation of the NBI_C index for the adaxial leaf side.

$$NBI_C\_R_{AD} = \frac{Chl_{AD}}{Flav_{AD}} = \frac{SFR\_R_T}{FLAV_{AD}} \tag{10}$$

$$NBI_C\_G_{AD} = \frac{Chl_{AD}}{Flav_{AD}} = \frac{SFR\_G_T}{FLAV_{AD}} \tag{11}$$

In Equations 12 and 13, the total NBI_T index of Equation 9 is rewritten explicitly for the red (R) and green (G) excitation in MX_H, respectively.

$$NBI_C\_R_T = \frac{Chl}{Flav} = \frac{SFR\_R_{AD} + SFR\_R_{AB}}{FLAV_{AD} + FLAV_{AB}} \tag{12}$$

$$NBI_C\_G_T = \frac{Chl}{Flav} = \frac{SFR_{\_G_{AD}} + SFR\_G_{AB}}{FLAV_{AD} + FLAV_{AB}} \tag{13}$$

On-the-go fluorescence sensor

The Multiplex On-The-Go (Multiplex 321 LD, FORCE-A) or mounted Multiplex, hereafter MX_M, is a Multiplex sensor adapted to be used mounted on an ATV or a tractor. It is synchronised with a global positioning system that allows for georeferencing of the fluorescence measurements. This device measures a surface of 10 cm diameter from a distance of approximately 20 cm. The fluorescence signals and indices provided by the MX_M are the same as those yielded by the MX_H. In this study, these indices included SFR, FLAV and NBI, among others. The leaves measured with the MX_M are a mix of AD and AB leaves, even though the prevailing exposed side will be the adaxial side of the leaf.

The SFR_R and the FLAV indices are calculated following Equations 4 and 6, respectively. In addition to NBI_R and NBI_G given by Equations 7 and 8, respectively, which coincide for both MX_H and MX_M, the NBI index (NBI_C) based on the ratio of SFR and FLAV indices of the MX_M was also calculated.

An exhaustive description of all formulae and equations of the fluorescence indices provided and calculated from the three sensors, DX4, MX_H and MX_M can be found in Table S1.

The comparison among these NBI indices would enable estimation of the error in the NBI provided by the MX_M with respect to the NBI given by the DX4, which is considered the reference. Towards that aim, the indices corresponding to the adaxial (named using AD as subscript) and abaxial (named using AB as subscript) sides of the

leaves, independently, were compared with the total (adaxial and abaxial) indices (named using T, for total, as subscript) for the whole leaf.

Fluorescence measurements

The 24 rows of the vineyard plot under study were manually monitored with the MX_H, the DX4 and on-the-go by the MX_M. For the manual devices, in each row, 13 sampling points, each one comprising three adjacent vines, were defined, at 10 m intervals. Measurements with MX_H and DX4 were conducted on the east side of the rows, on 12 leaves per sampling point (four leaves per vine). The same leaf was measured once with the MX_H and twice with the DX4, on both sides, abaxial and adaxial. The leaves measured with the hand-held devices were located at the mid-upper height of the canopy to satisfy the condition of being at the same height targeted and measured by the MX_M.

A total of 3744 manual measurements (24 rows × 13 sampling points × 12 leaves) on the abaxial and 3744 measurements on the adaxial sides of leaves with the MX_H, and 7488 measurement (24 rows × 13 sampling points × 12 leaves × two measurements) on each leaf side with the DX4 were taken. All rows were monitored on both sides of the canopy with the MX_M, mounted on an ATV moving at 5 km/h. The MX_M was placed 1.5 m above the ground, so that the leaves on the mid-upper part of the canopy (those same measured with the manual devices) were measured at a 20 cm distance. The acquisition rate for the MX_M was 60 Hz.

Data treatment and statistical analysis

The data obtained with the MX_H and MX_M devices were filtered by discarding readings higher than 4200 mV to avoid possible nonlinearity in the sensor response. On MX_H, the values lower than 10 mV, which correspond to the residual offsets, and the readings with a coefficient of variation of the FRF_R signal larger than 20% were also removed because this indicates that the sensor shifted during measurement acquisition or that fluctuations in variable Chl fluorescence were too large. On the MX_M, a histogram was computed to identify the data corresponding to leaves or canopy gaps. The latter were removed. After the filtering, the data of the two devices were standardised against a blue plastic-foil standard (Force-A) in order to compare the data obtained with other sensors and data collected under other measuring conditions. Prior to any statistical analysis, the data obtained with the three devices were corrected by applying the standard normal variate transformation to avoid the influence of measuring on different days (Legendre and Legendre 1998). Outliers were identified and excluded from the dataset by applying the Tukey method (Tukey 1977).

All three devices automatically provide the indices for the side from which the leaf is measured, adaxial or abaxial. In the case of DX4 and MX_H, to obtain calculated and total (T) leaf indices, the indices of the adaxial and the abaxial sides had to be added (cf. Equations 3, 9, 12 and 13). Indeed, each side of the leaf, either the palisade (adaxial) or the spongy mesophyll (abaxial), is different (Vogelmann and Evans 2002) and will have a fluorescence SFR and FLAV index specific to that side of the leaf. An exception is the CHL_T index of the DX4, which was used as the average of the adaxial and the abaxial sides (Equation 3) because the DX4 measures the Chl in transmittance mode; therefore, regardless the side from which the leaf is measured, the Chl index reflects Chl concentration of the whole leaf (Cerovic et al. 2012).

Once data were properly pretreated, the correlations between the same indices obtained by DX4 (reference method) and by the MX_H were computed and analysed. Correlations were separately calculated for the indices of the adaxial and abaxial sides of the leaf and for the total leaf indices.

The next step involved the calculation of the correlations between the MX_M indices and those obtained with the MX_H and DX4. For that purpose, as there were more data from the MX_M than from the two hand-held devices, and for different geographical coordinates, the data from each device were combined into a grid, generated by aggregation of the average of the nearest points (Figure 1). This grid allowed for having a common framework to analyse the correlations between MX_M, MX_H and DX4.

Classical global linear correlation models adjusted by ordinary least squares (OLS) were computed to analyse the

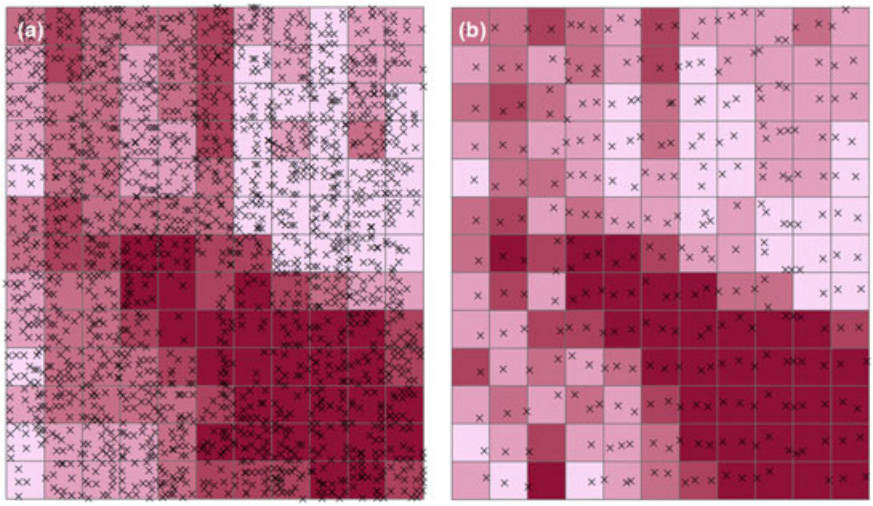

Figure 1. (a) Example of the grid generated to create a common framework for the Multiplex On-the-Go and (b) the reference, Dualex4 data.

relationships between the same indices measured with MX_M and with the two hand-held devices, MX_H and DX4. The strength and direction of the association were indicated by the determination coefficient (R^2). The RMSE and the coefficient of variation of the RMSE (%RMSE), calculated as the ratio between the RMSE by the mean value, were also computed to illustrate the robustness and accuracy of the predictions. Additionally, in order to provide an estimation of the accuracy of the MX_H and MX_M in predicting Chl, Flav and N concentration in grapevine leaves under field conditions, the total cumulative error $\%RMSE_{total}$ (Equation 14) was calculated, taking into account the %RMSE for each device against DX4 (Table 1), and the $\%RMSE_{validation}$ of DX4 against leaf extracts of Chl and Dualex3 measurements of Flav from the calibrations in Cerovic et al. (2012) and against leaf N concentration determined by wet chemistry (Cerovic et al. 2015).

$$\%RMSE_{total} = \sqrt{\%RMSE^2 + \%RMSE^2_{validation}} \quad (14)$$

Data pretreatment and statistical analysis were carried out using the softwares R (R Core Team 2012), Microsoft Office Excel 2013 (Microsoft Corporation, Redmond, WA, USA) and Statistica 9 (StatSoft., Tulsa, OK, USA).

Finally, the point-to-point measurements of the fluorescence indices of the three devices were interpolated to generate a continuous surface. Towards that end, the experimental variograms were computed and fitted the best model. The parameters of the fitted variograms, range, sill and nugget, were then used to apply the interpolation method of ordinary kriging. The software ArcGis 9.3 (ESRI, Redlands, CA, USA) was used for these geostatistical analyses.

Results

Calibration of the hand-held fluorescence sensor (MX_H) against the leaf-clip sensor (DX4)

Chlorophyll indices. The indices related to the Chl concentration in leaves were CHL in DX4 (reference) and SFR in MX_H. Figure 2 shows the correlations among these indices obtained for each side of the leaf (adaxial and abaxial) and for the whole leaf. Figure 2a evidenced no difference between CHL_{AD} and CHL_{AB} measured by DX4 ($n = 302$, $R^2 = 0.99$, $P < 0.001$, no offset). This was an expected result as DX4 works on transmittance mode for Chl; hence, regardless the side of the leaf that is measured the whole leaf Chl concentration is determined. From the results in Figure 2b, it can be seen that the indices SFR_R_T and SFR_G_T acquired with the MX_H were similar with an R^2 of 0.99 ($n = 302$, $P < 0.001$) and that a small bias in favour of G excitation was detected. Figure 2c,d shows a strong positive correlation between the SFR index, obtained from G and R excitation, respectively, with the CHL_T index, yielding R^2 of 0.93 ($n = 302$, $P < 0.001$) for SFR_G_T and R^2 of 0.92 ($n = 302$, $P < 0.001$) for SFR_R_T. In both cases, there was an offset of 10.4 (Figure 2c) and 15.3 (Figure 2d), which can be traced to the fluorescence reabsorption method used in the MX_H for the SFR index (FRF/RF). Figure 2e,f illustrates the fact that the correlations between the SFR index under red or green excitation from the adaxial side with the CHL_T index were equivalent to the correlations of CHL_T with the 'total' SFR_R_T (Figure 2d) or SFR_G_T (Figure 2c). These results indicate that the SFR indices from the adaxial side, which are reflecting the Chl concentration of the palisade mesophyll, are the component of the 'total' SFR_T index that mostly determined its variability among leaves; therefore, the SFR_{AD} can be used as a proxy of the leaf Chl concentration. Furthermore, when comparing the adaxial side with the abaxial side of the same SFR index (SFR_G or SFR_R), strong correlations were obtained with R^2 of 0.77 ($n = 302$, $P < 0.001$) (Figure 2g) and of 0.74 ($n = 302$, $P < 0.001$) (Figure 2h), respectively. A correlation matrix showing all possible relationships between the Chl related indices of DX4 and MX_H is included in Figure S1.

Flavonol indices. The fluorescence index FLAV is related to the leaf epidermal flavonols. Figure 3 shows the correlations between the FLAV indices measured with the two hand-held devices, DX4 and MX_H. With the DX4 measurements, $FLAV_{AB}$

Table 1. Calibration linear models for the fluorescence indices derived from the hand-held Multiplex and Multiplex On-the-Go sensors using the indices obtained with the leaf-clip, Dualex4.

Equations	Model parameters		Model statistics					
	Intercept	Slope	R^2	RSS	RMSE	BIAS	SEPC	% RMSE
CHL_T (DX4) = a + b*SFR_R_T (MX_H)	−15.32 (−16.60, -14.03)	10.76 (10.38, 11.12)	0.917	1286.298	2.064	0.038	2.064	10
CHL_T (DX4) = a + b*SFR_G_T (MX_H)	−10.41 (−11.47, -9.35)	8.96 (8.67, 9.25)	0.924	1165.282	1.964	0.007	1.964	9
$FLAV_T$ (DX4) = a + b*$FLAV_T$ (MX_H)	1.41 (1.28, 1.55)	0.90 (0.84, 0.95)	0.778	4.452	0.121	0.008	0.121	3
NBI (DX4) = a + b*$NBI_{C\_}R_T$ (MX_H)	−2.50 (−2.78, -2.20)	5.97 (5.78, 6.16)	0.926	126.432	0.647	0	0.647	11
NBI (DX4) = a + b*$NBI_{C\_}G_T$ (MX_H)	−1.63 (−1.89, -1.38)	5.15 (4.99, 5.31)	0.929	121.687	0.635	0	0.635	11
CHL_T (DX4) = a + b*SFR_R (MX_M)	−14.11 (−17.52, -10.69)	30.72 (27.78, 33.65)	0.752	1513.068	3.253	-0.002	3.253	15
$FLAV_T$ (DX4) = a + b*FLAV (MX_M)	1.58 (1.25, 1.91)	1.61 (1.36, 1.87)	0.520	3.323	0.152	0	0.152	4
NBI (DX4) = a + b*$NBI_{C_}$R (MX_M)	−1.89 (−2.62, -1.16)	8.63 (7.85, 9.41)	0.768	153.589	1.036	0	1.036	17
NBI (DX4) = a + b*NBI_R (MX_M)	0.78 (0.36, 1.38)	75.99 (68.87, 83.10)	0.760	163.261	1.069	0	1.069	18

The 95% confidence intervals for the fit coefficients are indicated in brackets. Intercept and slope coefficients were significant at $P < 0.001$ for all models. BIAS, bias; CHL, chlorophyll optical index; DX4, Dualex4; FLAV, flavonol optical index; MX_H, hand held Multiplex; MX_M, Multiplex On-the-Go; NBI, nitrogen balance index; RMSE, root mean square error; %RMSE, coefficient of variation of the RMSE calculated as the ratio of the RMSE to the mean value. ($n = 302$ for MX_H and $n = 143$ for MX_M); RSS, residual sum of squares; SEPC, standard error of prediction corrected for bias; SFR, simple fluorescence ratio.

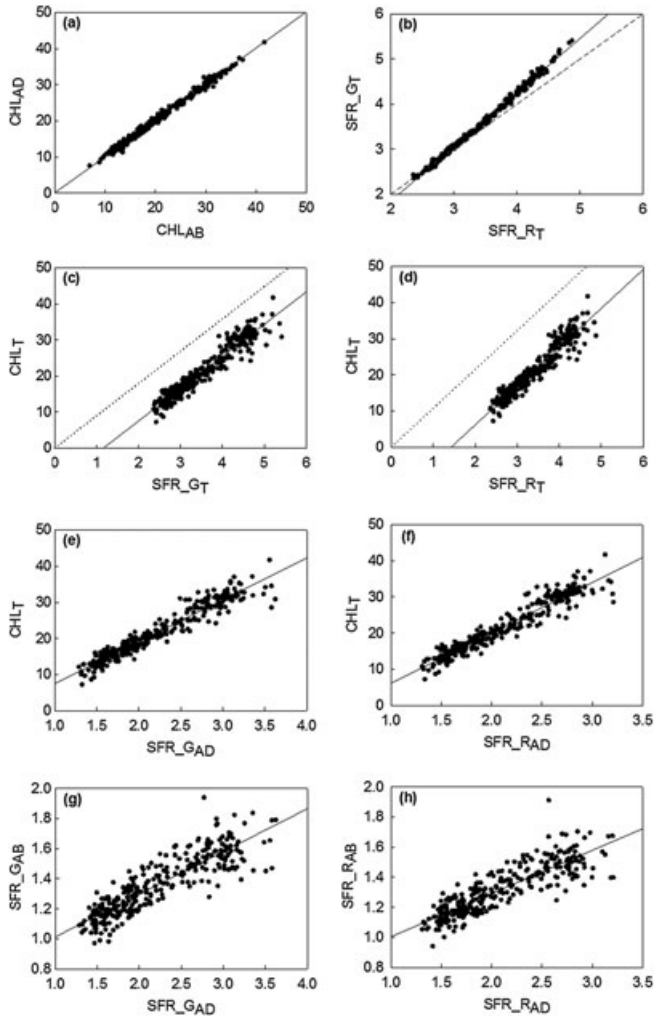

Figure 2. Correlations between the chlorophyll optical indices (CHL) and (SFR) related to the leaf chlorophyll concentration obtained with the hand-held Multiplex (MX_H) and Dualex4 (DX4) sensors. Coefficients of determination (a) $R^2 = 0.993$; (b) $R^2 = 0.996$; (c) $R^2 = 0.925$; (d) $R^2 = 0.917$; (e) $R^2 = 0.927$; (f) $R^2 = 0.920$; (g) $R^2 = 0.767$; (h) $R^2 = 0.743$ were significant at $P < 0.001$. $_{AD}$, measurement taken on the adaxial side of the leaf; $_{AB}$, measurement taken on the abaxial side of the leaf; $_T$, global index including measurements on adaxial and abaxial sides of the leaf; _R, fluorescence measurements using a red excitation source; and _G, fluorescence measurements using a green excitation source. Dashed line in (b) represents the 1:1 line. Dotted lines in (c) and (d) represent the regression lines of the same slope without the offset of the observed correlations. ($n = 302$).

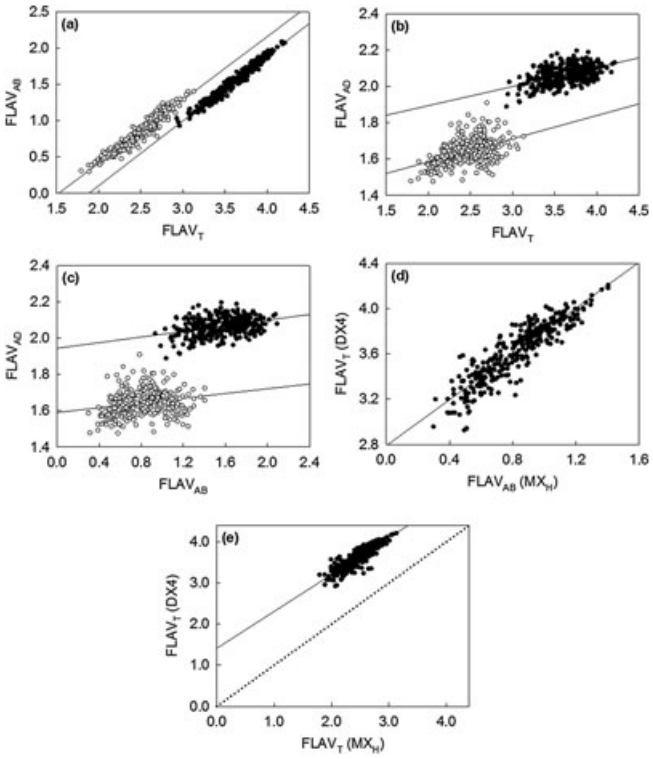

Figure 3. Correlations between the flavonols optical indices (FLAV) related to the leaf flavonols concentration obtained with the hand-held Multiplex (MX_H) (○) and Dualex4 (DX4) (●) devices. Coefficients of determination (a) $R^2 = 0.929$ (○), $R^2 = 0.964$, (●); (b) $R^2 = 0.270$ (○), R^2 0.220 (●); (c) $R^2 = 0.121$ (●), $R^2 = 0.047$ (○); (d) $R^2 = 0.815$ (●); (e) $R^2 = 0.779$ (●) were significant at $P < 0.001$. $_{AD}$, measurement taken on the adaxial side of the leaf; $_{AB}$, measurement taken on the abaxial side of the leaf; and $_T$, global index including measurements on adaxial and abaxial sides of the leaf. Dashed line in (e) represents the 1:1 line. $n = 302$.

yielded a strong correlation ($R^2 = 0.96$ at $P < 0.001$) with $FLAV_T$, which is the sum of $FLAV_{AD}$ and $FLAV_{AB}$ and represents the total amount of Flav in the leaf (Figure 3a), while $FLAV_{AD}$ correlated poorly with $FLAV_T$ ($R^2 = 0.27$ at $P < 0.001$) (Figure 3b) and $FLAV_{AB}$ (Figure 3c) owing to the reduced variation of Flav on this side of the leaf, which is more exposed to sunlight and tends to have a maximum epidermal Flav concentration. The same behaviour can be seen for the FLAV indices obtained with the MX_H (Figure 3a–c), where the Flav determined on the abaxial side of the leaf ($FLAV_{AB}$) appears to be those influencing the variation of the total leaf Flav ($FLAV_T$), with $R^2 = 0.93$ ($P < 0.0001$). This fact is also proved by the high correlation ($R^2 = 0.82$ at $P < 0.0001$) obtained between the Flav of the abaxial side of the leaf, measured by the MX_H, and the Flav of the whole leaf measured by the DX4 (Figure 3d). The relationship between $FLAV_T$ measured with DX4 and MX_H (Figure 3e) shows an offset of 1.4 absorbance units, which can be traced to the difference in wavelengths, and therefore extinction coefficients, for Flav used in DX4 and MX_H, which were 375 nm for DX4 and 385 nm for MX_H in the UV region and 650 nm for DX4 and 630 nm for MX_H in the red part of the spectrum. A correlation matrix showing all possible relationships between the FLAV indices of DX4 and MX_H is included in Figure S2.

Nitrogen indices. Two different NBI indices have been compared, the NBI index and the NBI_C index. The first is the NBI index provided directly by the MX_H, which is computed using Equations 7–9. The second (NBI_C) is an NBI index calculated for each side of the leaf or for the whole leaf (total NBI), using Equations 10–13, which are based on the formula given by Cartelat et al. (2005). The NBI_C index from the MX_H (Equation 9) is calculated following the same rationale as the NBI (DX4) in Equation 3, provided by the DX4, that is, the ratio of Chl-to-Flav.

Figure 4 shows the correlations among the indices related to the N status from the abaxial or the adaxial sides of the leaf as well as from the whole leaf, measured by the same device or between the two devices. The two ways of calculating the NBI index are taken into account, NBI_R and NBI_C_R indices, which are derived when red excitation was used. It should be noted that the global NBI_T index from the DX4 strongly correlated with either NBI_{AD} ($R^2 = 0.98$, $P < 0.001$) or NBI_{AB}

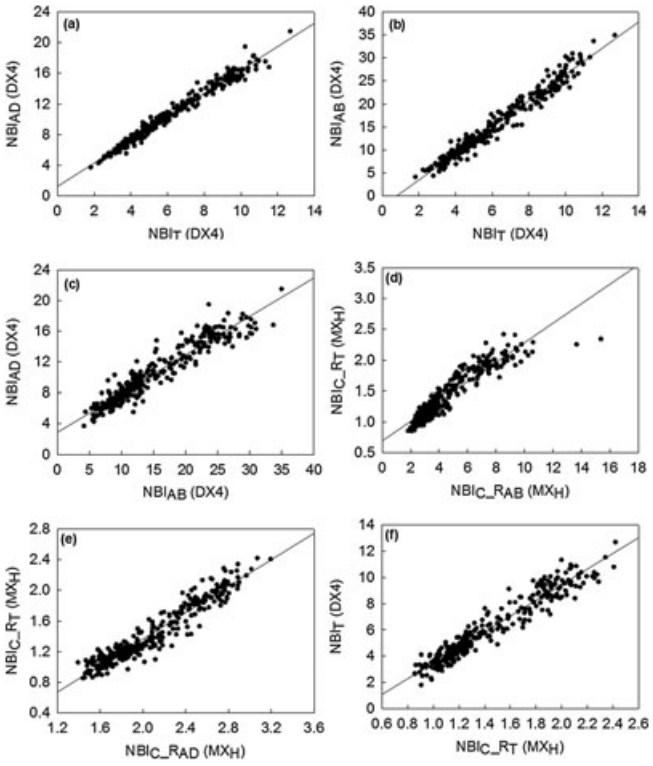

Figure 4. Correlations between the nitrogen balance (NBI) indices related to the leaf nitrogen balance obtained with the hand-held Multiplex (MX_H) and Dualex4 (DX4) devices. Coefficients of determination (a) $R^2 = 0.978$; (b) $R^2 = 0.966$; (c) $R^2 = 0.903$; (d) $R^2 = 0.874$; (e) $R^2 = 0.918$; (f) $R^2 = 0.926$ were significant at $P < 0.001$. $_{AD}$, measurement taken on the adaxial side of the leaf; $_{AB}$, measurement taken on the abaxial side of the leaf; $_T$, global index including measurements on adaxial and abaxial sides of the leaf; and _R, fluorescence measurements using a red excitation source. $n = 302$.

($R^2 = 0.97$, $P < 0.001$) of the same device (Figure 4a,b) and that the determination coefficient between NBI_{AD} and NBI_{AB} provided by the DX4 was also high ($R^2 = 0.90$, $P < 0.001$) (Figure 4c). Therefore, taking into account that the CHL index of DX4, either adaxial or abaxial, is measuring the total Chl concentration of the leaf, as stated before, and given that the equation to calculate the NBI index of the Dualex for the adaxial and the abaxial sides of the leaf is defined as the ratio of CHL-to-FLAV that corresponds to each side of the leaf (Table S1), the NBI index for adaxial or abaxial sides of the leaf is the ratio of the leaf Chl concentration of the whole leaf to the Flav concentration of the adaxial or abaxial sides of the leaf. This means that the Chl (represented by the CHL index in the numerator) is the component that is mostly determining the NBI value, because the flavonol concentration, represented by the FLAV index in the denominator, is the only component of the ratio that is different. A similar result for MX_H can be seen when analysing the correlations between the global $NBI_C\_R_T$ with $NBI_C\_R_{AB}$ ($R^2 = 0.87$, $P < 0.001$, Figure 4d) and $NBI_C\_R_{AD}$ ($R^2 = 0.92$, $P < 0.001$, Figure 4e). Indices $NBI_C\_R_{AB}$ and $NBI_C\_R_{AD}$ have been calculated as in the DX4, the numerator being global SFR_T and the denominator being the epidermal flavonols of either the abaxial ($FLAV_{AB}$) or the adaxial sides ($FLAV_{AD}$). The strong correlation of the total $NBI_C\_R_T$ with the indices of the two sides of the leaf, where again the only factor varying was the FLAV index, indicated that the Chl concentration (SFR index) was the component influencing the NBI index. The study of the relationships between the abaxial and adaxial versions of the NBI index provided by the MX_H (NBI_R_{AB} and NBI_R_{AD}, respectively) with the abaxial and adaxial versions of the calculated NBI_C index from MX_H ($NBI_C\_R_{AB}$ and $NBI_C\_R_{AD}$) revealed a strong significant correlation with R^2 higher than 0.88 at $P < 0.001$ (Figure S3). When the two NBI indices of the MX_H (NBI and NBI_C) were compared with the reference, a higher value of the determination coefficient corresponded to the $NBI_C\_R_T$ calculated as the Chl index (SFR_R_T) divided by the Flav index ($FLAV_T$) with an R^2 of 0.93 (Figure 4f), while the NBI_R_{AD} and the NBI_R_{AB} yielded determination coefficients of 0.75 and 0.67, respectively. Therefore, in the case of the MX_H, the more suitable NBI index for the assessment of the N status of the grapevine leaves would be the $NBI_C\_R_T$.

The NBI_G index was also analysed, and it yielded similar results (not shown) to the same index under red excitation (NBI_R). The correlation matrix, however, showing all possible relationships between the NBI indices of DX4 and MX_H when green excitation (_G) was used instead of red excitation (_R), is included in Figure S4.

The comparison of the indices retrieved with the MX_H and DX4 enabled the definition of the calibration equations (Table 1) to transform the SFR index (provided by MX_H) into absolute units of Chl and the FLAV index into absorbance units and to link the NBI_C (computed from the MX_H measurements) with the NBI index provided by DX4. The power to estimate the CHL, FLAV and NBI indices provided by the DX4 from MX_H measurements is given by the RMSE values in Table 1. Additionally, the accuracy was also estimated from the computation of the %RMSE, which ranged from 3 to 11% (Table 1). The $\%RMSE_{total}$ for the prediction of Chl, Flav and N concentration in grapevine leaves using the MX_H, under field conditions, was 18, 15 and 12%, respectively.

Calibration of the on-the-go fluorescence sensor (MX_M) versus hand-held devices

In order to ensure the reliability of the MX_M to assess the Chl, N and Flav concentration in grapevine canopies in a dynamic continuous way, calibration between the indices measured with the MX_M and hand-held devices is needed. The goal of this calibration is to be able to quantify the loss of explained variance (in terms or R^2) when the Multiplex sensor is used mounted on a vehicle instead of manually. With the two hand-held devices (DX4 and MX_H), both the abaxial and the adaxial sides of the leaf are accessible for measurements; therefore, indices for the whole leaf can be obtained. By contrast, the MX_M measures the vines from an ATV or a tractor in movement at approximately 20 cm from the canopy leaves. Under these conditions, leaves cannot be selected or manipulated. Therefore, the MX_M will target the leaves with the orientation that they show in the canopy at that precise moment of measurement. The adaxial side of the leaves will be exposed to the sensor most of the time, but there will be also some measurements from the exposed abaxial side of leaves.

The relationships between the MX_M indices and hand-held ones were studied applying the global regression model OLS. All the correlations were significant at $P < 0.001$ (Table 2). The calibration equations of MX_M against DX4 for CHL, FLAV and NBI are listed in Table 1. As with the MX_H, the RMSE values, illustrating the power to estimate the CHL, FLAV and NBI indices provided by the DX4 from MX_M measurements, are shown in Table 1, as well as the %RMSE values, which ranged from 4 to 18% (Table 1). The $\%RMSE_{total}$ for the prediction of Chl, Flav and N concentration in grapevine leaves

Table 2. Global linear models adjusted by ordinary least squares for the chlorophyll optical, flavonol optical and nitrogen balance indices studied derived from measurements with Multiplex On-the-Go versus hand-held Multiplex and the leaf-clip Dualex4 sensors.

Mounted Multiplex		Hand-held Multiplex			Dualex4		
		SFR_{AD}	SFR_{AB}	SFR_T		CHL_T	
SFR_R	R^2 (RMSE)	0.72 (0.24)	0.56 (0.97)	0.71 (0.32)		0.75 (3.25)	
		$FLAV_{AD}$	$FLAV_{AB}$	$FLAV_T$	$FLAV_{AD}$	$FLAV_{AB}$	$FLAV_T$
FLAV	R^2 (RMSE)	0.15 (0.049)	0.51 (0.14)	0.56 (0.14)	0.32 (0.035)	0.47 (0.15)	0.52 (0.15)
		NBI_R_{AD}	NBI_R_{AB}	—	NBI_{AD}	NBI_{AB}	NBI_T
NBI_R	R^2 (RMSE)	0.63 (0.0085)	0.62 (0.074)	—	0.75 (1.68)	0.74 (3.21)	0.76 (1.07)
NBI_C*_R*	R^2 (RMSE)	0.63 (0.0085)	0.59 (0.077)	—	0.77 (1.61)	0.74 (3.21)	0.77 (1.04)
		$NBI_C\_R_{AD}$	$NBI_C\_R_{AB}$	$NBI_C\_R_T$		—	
NBI_R	R^2 (RMSE)	0.69 (0.22)	0.72 (1.06)	0.74 (0.18)			
NBI_C*_R*	R^2 (RMSE)	0.71 (0.21)	0.72 (1.06)	0.74 (0.18)			

The root mean square error (RMSE) is shown in brackets for each relationship. All the coefficients of the model are significant at *P*-value < 0.001 ($n = 143$). $_{AD}$, indices measured only on the adaxial side of the leaf; $_{AB}$, indices measured only on the abaxial side of the leaves; CHL, chlorophyll optical index; DX4, Dualex4; FLAV, flavonol optical index; MX_H, hand held Multiplex; MX_M, Multiplex On-the-Go; NBI, nitrogen balance index; NBI_C, this index has been calculated as a division of SFR to FLAV, to differentiate it from the NBI provided by both Multiplex devices (see Table S1 in the Supporting Information); SFR, simple fluorescence ratio; $_T$, indices calculated for the whole leaf, abaxial and adaxial.

using the on-the-go MX_M was 22, 15 and 19%, respectively. In general, higher values of R^2 for Chl and NBI between the MX_M indices with those from MX_H and DX4 corresponding to the adaxial side in comparison with those from the abaxial side were observed, indicating that the MX_M was mainly seeing the adaxial side of the leaves, as expected. The exception was FLAV because the adaxial FLAV had a small span, as mentioned for the correlations of the hand-held devices (Table 2). The Chl-related SFR index obtained on-the-go with the MX_M proved to be well correlated with global SFR_T from MX_H ($R^2 = 0.71$ at $P < 0.001$), and CHL from the reference DX4 ($R^2 = 0.75$ at $P < 0.001$). Slightly improved correlations were found for the NBI indices (with R^2 between 0.74 and 0.77 at $P < 0.001$), but more moderate correlations were found for FLAV (R^2 of 0.56, $P < 0.001$) (Table 2).

Comparing the relationships between the SFR_T index from MX_H and the SFR index from MX_M with the CHL_T from the reference DX4, better and more accurate (lower RMSE and %RMSE, Table 1) correlations were obtained for the MX_H device ($R^2 = 0.92$, $P < 0.001$, Figure 2d) than for MX_M ($R^2 = 0.75$, $P < 0.001$, Table 2). These results indicate that a loss of nearly 20% of information occurred when the Multiplex operated on-the-go. For the FLAV index, the loss of explained variability when measuring on-the-go instead of manually with the MX_H, was about 13%, although the accuracy of the results remained similar, between 3 and 4%, in terms of %RMSE (Table 1). The same comparison showed a loss of 15% for the NBI_R_T or the $NBI_C\_R_T$ indices. For these indices, the accuracy diminished with the MX_M (%RMSE =17–18%) (Table 1), but these can be considered fairly good figures for non-destructive, on-the-go measurements.

Spatial variability

Figure 5 depicts the krigged maps for the global indices of the three sensors. The maps showed a similar spatial distribution for the three indices studied, independently of the device used. Different areas could be clearly identified. In the case of the Chl and N, a higher value can be seen in the upper-right (northeast) part of the plot, and at the left (west) part of the plot, there can be seen two vertical rows following a double and a single grapevine row that were identified as Tempranillo and Grenache rows; the highest values are located at the bottom-right (southeast) part of the plot with a narrower part going towards left, this irregular shape perpendicular to the grapevine rows direction followed a sharp change in soil characteristics, regardless the grapevine cultivar or clone planted. Flav followed an inverse spatial distribution relative to that of Chl and N.

Discussion

Optical sensing systems are suitable tools to provide frequent and spatially widespread monitoring of plant nutrient status (Muñoz-Huerta et al. 2013). This paper presents the first calibration of the fluorescence-based Multiplex sensor, used manually (MX_H) and on-the-go (MX_M), on grapevine leaves against the Dualex4 as the reference. In this work, the MX_H and MX_M have been studied to determine their capability to satisfactorily measure, in the case of the MX_H, and to estimate, in the case of the on-the-go MX_M, the grapevine's leaf Chl, epidermal Flav and N concentration.

The DX4 was chosen as the reference for several reasons. First, its performance to yield a reliable and accurate measurement of the Chl concentration (Cerovic et al. 2012), epidermal Flav (Agati et al. 2008, Cerovic et al. 2012) and N concentration (Cerovic et al. 2015) has been shown even in grapevine leaves. Second, the efficiency of leaf extraction by organic solvents may be a potential problem for the calibration of sensors (Lashbrooke et al. 2010) as well as the operator's skill, which is a major source of variability (Cerovic et al. 2012). Casa et al. (2015) reported higher average coefficient of variation values for Chl assessment using wet chemistry methods than using DX4 in four different crops.

The MX_H has been used for the study of leaf Flav (Agati et al. 2011b, Müller et al. 2013) and N status of different species,

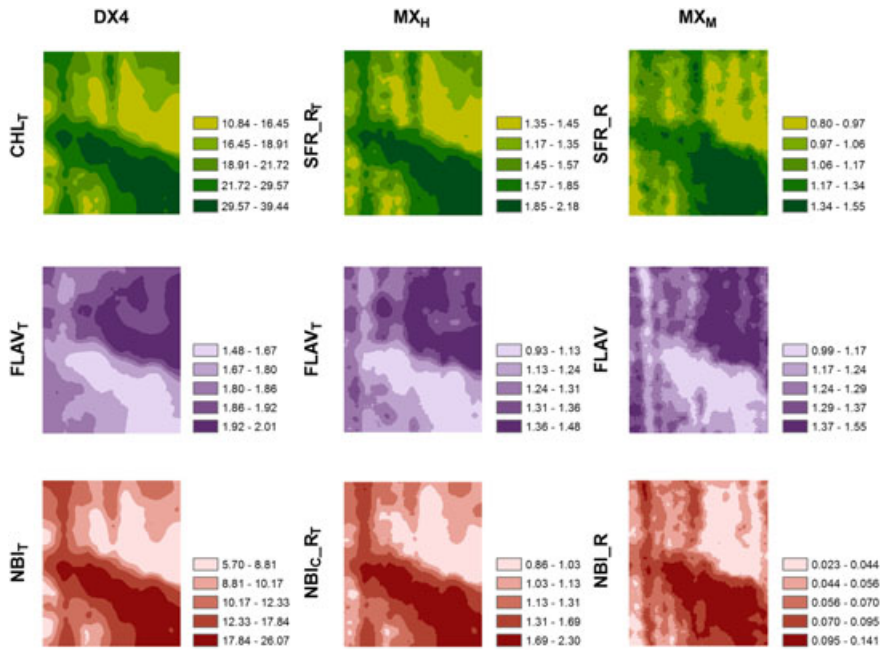

Figure 5. Interpolated surfaces by ordinary kriging of chlorophyll indices (CHL and SFR), epidermal flavonol index (FLAV) and nitrogen balance index (NBI) acquired by Dualex4, hand-held Multiplex and Multiplex On-the-Go sensors. Maps were represented by quantiles.

such as potato (Ben Abdallah and Goffart 2012), turf-grasses (Agati et al. 2013b), rice (Li et al. 2013) and maize (Zhang and Tremblay 2010, Zhang et al. 2012, Longchamps and Khosla 2014). While its application on vineyards has been widely focused on grape bunches to assess their anthocyanin concentration (Ben Ghozlen et al. 2010, Baluja et al. 2012, Agati et al. 2013a), few studies have been carried out on grapevine leaves (Serrano et al. 2011). In other crops, the only work reporting the assessment of Chl by MX_H was conducted by Tremblay et al. (2012), who validated the SFR_G_{AD} index against Chl extractions for kiwi leaves. In the present study, the two SFR indices (_R or _G) have been calibrated against the CHL_T index provided by the DX4 and the calibration equations for the global SFR_R_T and SFR_G_T are obtained. This means that the SFR index provided by the MX_H can now be translated, going through DX4 units, into Chl units for grapevine leaves.

To be able to calculate the Chl and Flav indices at the whole leaf level, the two sides of the leaf have been measured. This is important as the incident light does not penetrate into the entire leaf depth; therefore, the emitted Chl fluorescence would correspond to either the part of the leaf closer to the adaxial side or the part of the leaf closer to the abaxial side, that is, the palisade or spongy mesophyll (Koizumi et al. 1998, Karabourniotis et al. 2000, Vogelmann and Evans 2002). The evidence of correlations between SFR_{AB} and SFR_{AD} in Figure 2g,h would enable the calculation of one of them (i.e. SFR_{AB}) from the other one (SFR_{AD}), and in this way, the calibration and correlation with the MX_H and MX_M data would be simplified by using only $SFR_{AD.}$ Indeed, adding the SFR_{AB} data to SFR_{AD} data does not improve the correlation with CHL_T obtained by DX4, but it does not make it worse either (Figure S1). The correlation of SFR_{AD} ($R^2 = 0.920$ and 0.927, for R and G, respectively) with CHL_T (DX4) is much better than SFR_{AB} ($R^2 = 0.726$ and 0.752, for R and G, respectively). It is especially evident for the higher leaf Chl concentration (above 25 $\mu g/cm^2$, Figure S1) present on the southeast part of the plot, in which the SFR_{AD} versus SFR_{AB} correlation is also poorer (Figure 2g). This is the first publication, however, of MX_H calibration for the assessment of the Chl concentration. It would be better if it could be more generally applicable to all species and Chl distribution: dicots, monocots, homogeneous or heterogeneous Chl distribution. This is why we favour the sum of both sides of SFR. The major difference of the fluorescence-ratio technique for Chl assessment compared with transmission-based techniques is that it can reveal the differences in dorso-ventral distribution of leaf Chl.

In addition to the Chl-related SFR index, the FLAV index has also proved to be able to assess the epidermal Flav concentration of grapevine leaves.

On-the-go monitoring of the relevant parameters of a vineyard related to the vigour and nutritional status of a plant in a non-destructive, fast, and reliable way would enable the mapping and characterisation of the spatial-temporal variability of these variables. This information can help to: (i) optimise vineyard management (reduction of inputs and other management costs and application of variable fertilisation rates) and making it more sustainable; (ii) identify homogeneous management zones within a vineyard; and (iii) improve the quality of grapes and wine. For these reasons, the calibration and evaluation of performance of the on-the-go MX_M device against a widely verified reference, such as the DX4, to characterise the Chl, epidermal Flav and N concentration in grapevine leaves, were attempted.

The results obtained in the present work showed that the fluorescence indices measured with the MX_M successfully explained a significant fraction of the variance of the same indices measured by the DX4, therefore confirming the capability of the Multiplex sensor operating on-the-go to assess the Chl, epidermal Flav and N concentration in grapevine leaves. Several factors can affect the fluorescence measurements made in a continuous way with the $MX_{M.}$ All involve leaf features, such as the side of the leaf exposed to the sensor, leaf exposure to sunlight during growth and leaf age (i.e. leaf position on the shoot). These three factors are less controlled in on-the-go operations in comparison with manual measurements, as in the latter, the leaves to be measured are susceptible to be chosen and detected under a perpendicular geometry. These factors

are additional sources of uncertainty for predicting Chl, Flav and N concentration in grapevine leaves with the MX_M compared with that of the MX_H and responsible for the diminishment in the accuracy observed. Nevertheless, values of $\%RMSE_{total}$ around 20% for the estimation of these three leaf constituents with the MX_M, which measures non-destructively and continuously from a vehicle moving at 5 km/h, are satisfactory and enable the delineation of zones of homogeneous nutritional status and vigour within a given vineyard. This is the ultimate goal and most powerful capability of such an on-the-go sensor, as it can efficiently provide helpful and robust enough information to the grapegrower from the acquisition of a large amount of measurements needed for mapping.

The MX_M indices, even though they are mainly from the adaxial side, were highly correlated to those from the reference DX4. Light exposure of the measured leaves affects the fluorescence indices indirectly, by impacting the leaf mass per area (LMA). Sun-exposed leaves tend to be thicker than shaded leaves and possess a higher concentration in surface-based Chl (Lichtenthaler et al. 2007). The NBI index, because it is defined as the Chl-to-Flav ratio – the latter being a surrogate of the LMA (Meyer et al. 2006) – will not be affected by the increase in LMA. The third effect that influences the MX_M measurements would be the leaf age, as the sensor is measuring the leaves on both primary and secondary (lateral) shoots. Leaves on primary shoots are older and thicker than those on secondary shoots. Similarly to the light exposure effect, the NBI, which is a ratio of Chl and Flav, will be less affected by the leaf age than simple surface-based indices such as SFR and FLAV.

The indices related to the epidermal concentration of Flav and Chl are valuable for N management (Tremblay et al. 2012) as they allow the calculation of the NBI index. The latter, as an indicator of the N status of the plant, has been analysed here by comparing different sensors and ways to calculate it. The NBI_C is based on the inverse dependence of N on Chl and Flav, which increases the dynamic range, lessens the leaf position and exposure influence and, finally, voids the effect of the LMA. In contrast, the NBI index provided by MX_H or MX_M is not a ratio of the Chl to the Flav concentration but the ratio of two signals (FRF and RF), and it can be obtained for either the adaxial or the abaxial sides of the leaf but not for the whole leaf. Besides these differences in the equations defining the NBI index, the NBI provided directly by the MX_M was equally good as the calculated NBI_C, of the same device compared with that of the DX4 reference. This finding has important practical implications when using the MX_H and MX_M devices. For the MX_H, the NBI_C would be a more accurate index of the N status of the plant, while when MX_M is employed, both ways of calculating the N balance index can be equally used to estimate the variation in the N status within the vineyard.

Efficient mapping of leaf attributes was demonstrated in this work. Therefore, in the framework of precision farming, the MX_M enables a fast, non-destructive, reliable on-the-go assessment of the spatial and temporal variability (as several measurements may be conducted during the season) of important indicators of the vegetative and nutritional status of the grapevine. In this way, detection of chlorotic vines, susceptible to additional iron or other mineral amendments, may be carried out early in the season, and objective appraisal of the recovery of the plants after mineral spraying can be performed with the MX_M. The MX_M also allows the assessment of the total Flav, which provides information about the exposure of leaves to light and their potential susceptibility to diseases or pathogens (Agati et al. 2008, 2013b).

The krigged sufaces have revealed that the indices measured using the Multiplex device, manually and on-the-go, showed the same spatial variability as the indices measured by the DX4. Both MX_H and MX_M were also able to bring out the soil variability within the vineyard plot and its effect on the vegetative growth of the plant, as well as some variation induced by the nature of the planted grapevine cultivar, just as did the reference DX4. The sharp soil change detected in the south middle part of the plot was obvious during field measurements. This change in the soil characteristics was certainly affecting the leaf Chl, Flav and N concentration (Van Leeuwen 2010); therefore, it was expected to be prompted by the fluorescence indices.

Diagnosis of plant N status is valuable for rational management of N in a sustainable fertilisation context. While the MX_M allows a rapid estimation of the N status distribution within the vineyard and delineation of homogeneous subzones for the application of variable rate fertilisation strategies, precise quantification of the N concentration should be conducted either with MX_H or DX4 once the homogeneous management zones have been defined.

Overall, the Multiplex sensor, used manually or on-the-go, may be used as a reliable phenotyping tool. In the case of the MX_M, phenotyping can be carried out in a faster and more continuous way, enabling a rapid, reliable and non-destructive assessment of the spatial variability of the vegetative and nutritional status within a vineyard at several times within the season.

Conclusions

Optical sensors are capable of providing numerous and spatially widespread monitorings of plant nutrient status in comparison with destructive time-consuming wet chemistry analyses. An exhaustive calibration of the fluorescence-based Multiplex optical sensor, used either manually or on-the-go, was conducted against the reference Dualex sensor for the first time, to assess the Chl and Flav concentration as well as the N status of grapevine leaves.

The capability to satisfactorily measure the nutritional and vegetative attributes of grapevines using the Multiplex was demonstrated through the defined calibration equations between the MX_H or the MX_M and the DX4. From the on-the-go approach, the fluorescence indices measured with the MX_M successfully explained a high fraction of the variance of the same indices measured by the DX4, hence confirming the capability of the Multiplex used on-the-go to estimate the Chl, epidermal Flav and N concentration in grapevine leaves in motion. Of the several, not-controlled factors in on-the-go operations, potentially affecting the performance of the MX_M, the side of the leaf exposed to the sensor did not appear to alter any of the fluorescence indices obtained, while leaf exposure to sunlight during its growth and leaf age were found to influence more the simple surface-based indices such as SFR and FLAV than the ratio-based NBI.

Non-destructive, on-the-go monitoring of key parameters of a vineyard related to the vigour and nutritional status of the plant with the MX_M will enable the mapping and characterisation of the spatial-temporal variability of these parameters. This information will be valuable to support decision-making for optimised vineyard management as well as to delineate homogeneous zones within the vineyard, in the frame of precision viticulture.

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Conflict of interest

The authors declare the following competing financial interest(s): Mr Z. C. Cerovic 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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Table S1. Nomenclature and equations of the different chlorophyll, flavonol and nitrogen balance indices provided by the Dualex4 (DX4), hand-held Multiplex (MX_H) and Multiplex On-the-Go (MX_M) sensors and calculated from the provided indices.

Figure S1. Correlation matrix between the chlorophyll indices of the hand-held Multiplex (MX_H) and the leaf-clip Dualex4 (DX4). CHL_{AD} and CHL_{AB} are the chlorophyll indices for adaxial and abaxial sides of the leaf, respectively, given by the DX4 device, and CHL_T is the sum of the adaxial and abaxial sides. The simple fluorescence ratio (SFR_G and SFR_R) is the chlorophyll index yielded by the MX_H. Adaxial (SFR_R_{AD} and SFR_G_{AD}) and abaxial (SFR_R_{AB} and SFR_G_{AB}) sides are also taken into account and the index of the whole leaf as the sum of each side of the leaf. Each combination R^2 is indicated in the right side of the graphic diagonal, and all of them are statistically significant at $P < 0.001$ ($n = 302$).

Figure S2. Correlation matrix between the epidermal flavonol indices of the hand-held multiplex (MX_H) and the leaf-clip Dualex4 (DX4). $FLAV_{AD}$ and $FLAV_{AB}$ are the epidermal flavonol indices for adaxial and abaxial sides of the leaf, respectively, and $FLAV_T$ is the sum of the adaxial and abaxial sides. Each combination R^2 is indicated in the right side of the graphic diagonal, and all of them are statistically significant at $P < 0.001$ ($n = 302$).

Figure S3. Correlation matrix between the nitrogen balance indices of the hand-held multiplex (MX_H) and the leaf-clip Dualex4 (DX4). NBI_R_{AD} and NBI_R_{AB} are the nitrogen balance indices for adaxial and abaxial sides of the leaf, respectively. $NBI_C\_R_{AD}$ and $NBI_C\_R_{AB}$ are the nitrogen balance indices calculated as SFR_R divided to $FLAV_{AD}$ or $FLAV_{AB}$, respectively. Each combination R^2 is indicated in the right side of the graphic diagonal, and all of them are statistically significant at $P < 0.001$ ($n = 302$).

Figure S4. Correlation matrix between the nitrogen balance indices of the hand-held multiplex (MX_H) and the leaf-clip Dualex4 (DX4). NBI_G_{AD} and NBI_G_{AB} are the nitrogen balance indices for adaxial and abaxial sides of the leaf, respectively. $NBIC\_G_{AD}$ and $NBIC\_G_{AB}$ are the nitrogen balance indices calculated as SFR_G divided to $FLAV_{AD}$ or $FLAV_{AB}$, respectively. Each combination R^2 is indicated in the right side of the graphic diagonal, and all of them are statistically significant at $P < 0.001$ ($n = 302$).