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Geographical discrimination of grapevine leaves using fibre optic fluorescence data and chemometrics. Determination of total polyphenols and chlorophylls along different vegetative stages

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ABSTRACT

Auto fluorescence of grapevine leaves has been employed to discriminate vineyards from two different geographical regions and to quantify the amount of total polyphenols and chlorophylls. For that, fluorescence spectroscopy with an optical fibre was used on samples of grapevine leaves (previously freeze-dried) and excitation-emission matrices were collected. Two spectral regions were analysed independently: one with excitation wavelengths from 279 to 372 nm and emission wavelengths from 384 to 496 nm, and the second with excitation between 390 and 585 nm and the emission between 654 and 756 nm. Firstly, an exploratory analysis was performed with parallel factor analysis (PARAFAC) to extract useful information from the data (scores and loadings). The obtained scores were used to perform a linear discriminant analysis (LDA) model for the classification of samples according to its geographical region. A total of 95% of correct predictions were obtained in the validation samples. Moreover, the PARAFAC scores of the components used in each model were analysed along the vegetative stage and a clear evolution with time has been observed.

The quantification of total polyphenols and chlorophylls was also carried out with unfolded partial least squares (U-PLS) and N-way partial least squares (N-PLS). Good correlation coefficients (for the validation set) were obtained for both parameters and similar results were achieved with both algorithms (0.82 (U-PLS) and 0.76 (N-PLS) for total polyphenol and 0.83 (U-PLS) and 0.82 (N-PLS) for total chlorophylls).

1. Introduction

Grapevine leaves are an abundant source of bioactive compounds, mainly phenolic compounds, which are connected to beneficial properties in the human body. Polyphenolic compounds are the most popular healthy compounds found in vegetables. The inclusion of vine leaves in the human diet could be a good strategy to enhance the use of this by-product of the vine. In fact, certain cultures of the Mediterranean basin have traditionally consumed them [1].

When studying the composition of grapevine leaves, several phenolic compounds are detected, namely chlorogenic acid, quercetin, quercetin-3-O-glucoside, quercetin-3-O-glucoside-7-O-glucronide, isorhamnetin-3-O -glucoside and kaempherol-3,7-O-diglycoside [2]. In general, the polyphenols concentration and its profile in grapevine leaves depend on different factors such as the cultivar, the agroclimatic conditions (also known as “terroir”), the agronomic techniques and the maturation stage [3]. Therefore, it is important to study their concentration along different vegetative stages.

Besides these compounds, there is another group of compounds present in grapevine leaves, namely chlorophylls, that are worth to study. In fact, the content of these compounds (chlorophylls *a* and b) as well as their degeneration products (pheophytin a and b) are good indicators about the maturation state of the plant. Moreover, it is common knowledge among grapevine producers that grapevine leaves are a good indicator of the vigor, water stress and presence of diseases in the vine and, consequently, can be used to diagnose the plant status [4,5].

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Therefore, the development of rapid, reliable and environmental friendly analytical techniques capable of quantifying the content of polyphenolic and chlorophyll compounds in grapevine leaves could enhance the use of this material as a food source as well as to help the producers to maintain healthy plants. Moreover, in food science, there is a growing need for analytical methods capable of performing the characterization of foods and the identification of the most relevant compounds. In this sense, it is very important to have scientific tools that can provide an objective and reliable characterization of plant products in a rapid way. Moreover, as the origin of plant products is connected to their quality and therefore its economic value, it is important to possess analytical tools capable of attesting and assessing their origin.

Several spectroscopy techniques have been applied to analyse intact food systems, namely in grapevine plants. Vis/NIR spectroscopy allowed to determine anthocyanin content in grapevine leaves in two French hybrid vine cultivars collected in two different dates [6]. Gutierrez *et al.* (2015) used a portable NIR instrument for in-field grapevine varieties discrimination using the leaves spectra [7].

More recently, near infrared spectroscopy (NIRS) and mid-infrared spectroscopy (MIRS) coupled to chemometrics were applied to discriminate the geographical origin of grapevine leaves during different vegetative stages in two different wine regions of Portugal. Both, NIRS and MIRS techniques demonstrated that it is possible to discriminate between the two geographical origins with an outstanding accuracy. Moreover, the quantification of total chlorophyll and total polyphenol content from grapevine leaves spectra was also attempted by both techniques. The quantification of chlorophyll content yielded good results while the total amount of polyphenols was not satisfactory assessed. Moreover, when comparing both techniques, NIR technique yielded the best results. This work revealed that it is possible to track the chemical evolution of grapevine leaves over the ripening period, leaving an open possibility to use this methodology for monitoring the grapes ripening process [8].

Both, polyphenol and chlorophylls compounds are highly fluorescent. In fact, the fluorescence of grapevine leaves is due to, in large extent, both types of these compounds, although different ones could also contribute to the total fluorescence. The fluorescence profiles of grapevine leaves are affected by environmental conditions and agronomic practices and therefore, could be related with the geographical region of cultivation and harvest season [9]. In this sense, the use of the fluorescence properties of grapevine leaves in combination with multivariate data analysis has a huge potential for food traceability as well as for helping grapevine producers to monitor the grapevine status.

However, fluorescence spectroscopy has hardly ever been used with that purpose and as far as we know, the application of fluorescence spectroscopy with an optical fiber has never been used in grapevine leaves.

Moreover, three-way fluorescence data has significant advantages over one-way and two-way data for characterization and classification [10]. The data provide by three-way fluorescence data can be analysed by second order algorithms which allows to obtain the second-order advantage. Therefore, it is possible to quantify the analytes of interest in the presence of uncalibrated species.

Unsupervised and supervised methods are commonly applied to extract spectral characteristics and develop classification models. Among the supervised monitoring methods, it can be highlighted parallel factor analysis (PARAFAC) algorithm [11], which is a generalization of principal component analysis (PCA) for a set of data matrices. It is used to decompose trilinear data with a single solution, enabling robust estimates of excitation and emission profiles present in the spectra and their concentrations. The decomposed data coming from PARAFAC can be used to build the LDA classification model. Recently, a publication about modeling second-order data for classification issues has been published [12].

Regarding to quantification, the most employed chemometric algorithms for fluorescence excitation emission matrices (EEMs) are PARAFAC, unfolded partial least squares (U-PLS) and N-way partial least squares (N-PLS) [13]. General information about second-order multivariate calibration has been widely studied [14].

The main goal of this work was to demonstrate that solid state fluorescence spectroscopy is suitable for grapevine leaves analysis along different vegetative stages, in terms of monitoring grapevine plant status (chlorophyll quantification) as well as of enhancing the consumption of grapevine leaves (polyphenol quantification). Moreover, this technique was also applied for the discrimination between two different geographical regions to assess and attest their origin. For this purpose, EEMs were obtained from the solid powder of grapevine leaves without any chemical treatment using a fluorescence probe. The leaves were collected from two different geographical regions and at four different months across its vegetative cycle.

2. Material and methods

2.1. Sample description

Samples of grapevine leaves, all of them belonging to plants of the same variety ("Touriga Nacional"), were analyzed. Each collected sample is composed by twenty leaves, harvested from five different plants. Samples were collected from two vineyards, located in two Portuguese wine regions: *Quinta dos Carvalhais* (QC) in the Dão Wine Region and *Quinta da Leda* (QL) in the Douro Wine Region, property of SOGRAPE VINHOS SA. Eight different geographical points were sampled in each vineyard, and in each one of them, leaves were collected over four time periods, with approximately one-month interval, from June to September (ripening period) during the 2017 campaign. Therefore, a total of 64 samples were obtained, 32 samples from QC and the other 32 samples from QL (8 samples of each month).

After sampling, leaves were transported to the laboratory and stored in the freezer (-20 °C) until lyophilization. Leaves were lyophilized at −80 °C and 0.4 mbar during 3 days in a Telstar, Lyoquest 85, lyophiliser. Once lyophilized, all leaves from each sampling point were mixed and milled. The samples were then stored at room temperature in the dark until analysis.

2.2. Instrumentation and software

Fluorescence measurements were performed on a Cary Eclipse VARIAN spectrofluorometer equipped with two Czerny–Turner Monochromators (excitation and emission), a xenon light source and a photomultiplier tube as detector. Measurements were performed with an optical fiber-probe in direct contact with the solid samples. The Cary Eclipse 1.0 software was used for data acquisitions.

EEMs were measured in two different spectral regions: in the first spectral region (R1), the excitation ranged from 279 to 372 nm (each 3 nm), and the emission ranged from 384 to 496 nm (each 2 nm); and in the second spectral region (R2) the ranges were 390–585 nm (each 3 nm, excitation), and 654–756 nm (each 2 nm, emission). The slits of excitation and emission monochromators were set at 5 nm. The photomultiplier tube sensitivity was 800 V and 650 V for the first and second region, respectively. Moreover, emission spectra were smoothed using the Savitzky–Golay filter (5 experimental points).

2.3. Chemometric analysis

Data analysis has been performed using the graphical interface MVC2 [15] in Matlab environment (Matlab R2016b, The Marhworks, Natick, Massachusetts, USA) and an in house MatLab routine was used for linear discriminant analysis (LDA) calculations [16].

Firstly, with the aim of exploring the main variation between the groups, an exploratory analysis was performed with the aid of parallel factor analysis (PARAFAC) [11]. Core consistency diagnostic criterion (CORCONDIA) [17], residual analysis [11] and the physiognomy of the

loadings were employed to select the optimum number of components. Non-negative constraints were applied to all models with the objective of obtaining a realistic solution as all concentrations and spectral values are always positive.

After that, to evaluate the possibility of discriminating samples according to their geographical region and sampling date, LDA was applied to the PARAFAC scores [18].

To develop quantitative models between EEMs and chemical parameters, unfolded partial least squares (U-PLS) and N-way partial least squares (N-PLS) regressions were used [13]. The optimum number of components were selected through cross validation, following Haaland and Thomas criterion [19]. The optimal number of components is indicated when a PRESS value that is not statistically different to the minimum PRESS value (F-ratio probability falling below 0.75) is achieved.

The samples were divided into two sets to perform the classification/quantification models. The first data set (training set) was composed by the 70% of the samples, randomly selected. The training set was used to perform the calibration and the cross validation of the models. The other dataset (test set) was composed by the remaining samples (30% of the total samples) and it was used to test the robustness and accuracy of the developed models.

2.4. Chemical analysis

2.4.1. Total polyphenol determination

Total polyphenol content was determined according to the Folin-Ciocalteu colorimetric method [20] using a Cary 50 UV–VIS spectrophotometer (Agilent Technologies). For the polyphenol extraction, 0.25 g of the milled samples were mixed with 10 mL of methanol:water (80:20) in an ultrasound bath during 30 min. The extract was centrifuged for 10 min at 3000 rpm. The supernatant was diluted 1:33 (v:v) with ultrapure water.

One millilitre of the diluted extract, or gallic acid standard, was mixed with 5 mL of Folin-Ciocalteu reagent (1:10 v/v with water) and 4 mL of 75 g L^{-1} sodium carbonate solution. The mixture was incubated for 1 h at room temperature and the absorbance was measured at 760 nm against a blank solution. External standard calibration was used. The total polyphenol content was expressed as mg of gallic acid per g of lyophilized leaves.

2.4.2. Total chlorophyll determination

For the chlorophylls extraction, 0.1 g of the lyophilized leaves were mixed with 10 mL of methanol in centrifuge tubes using a vortex. Tubes were placed in an ultrasound bath for 15 min and centrifuged at 3000 rpm for 10 min. Then, 300 μL of the supernatant were diluted to 3 mL with methanol. The absorbance of this solution was measured at 652 and 665 nm and the concentrations of chlorophyll *a* and b were determined

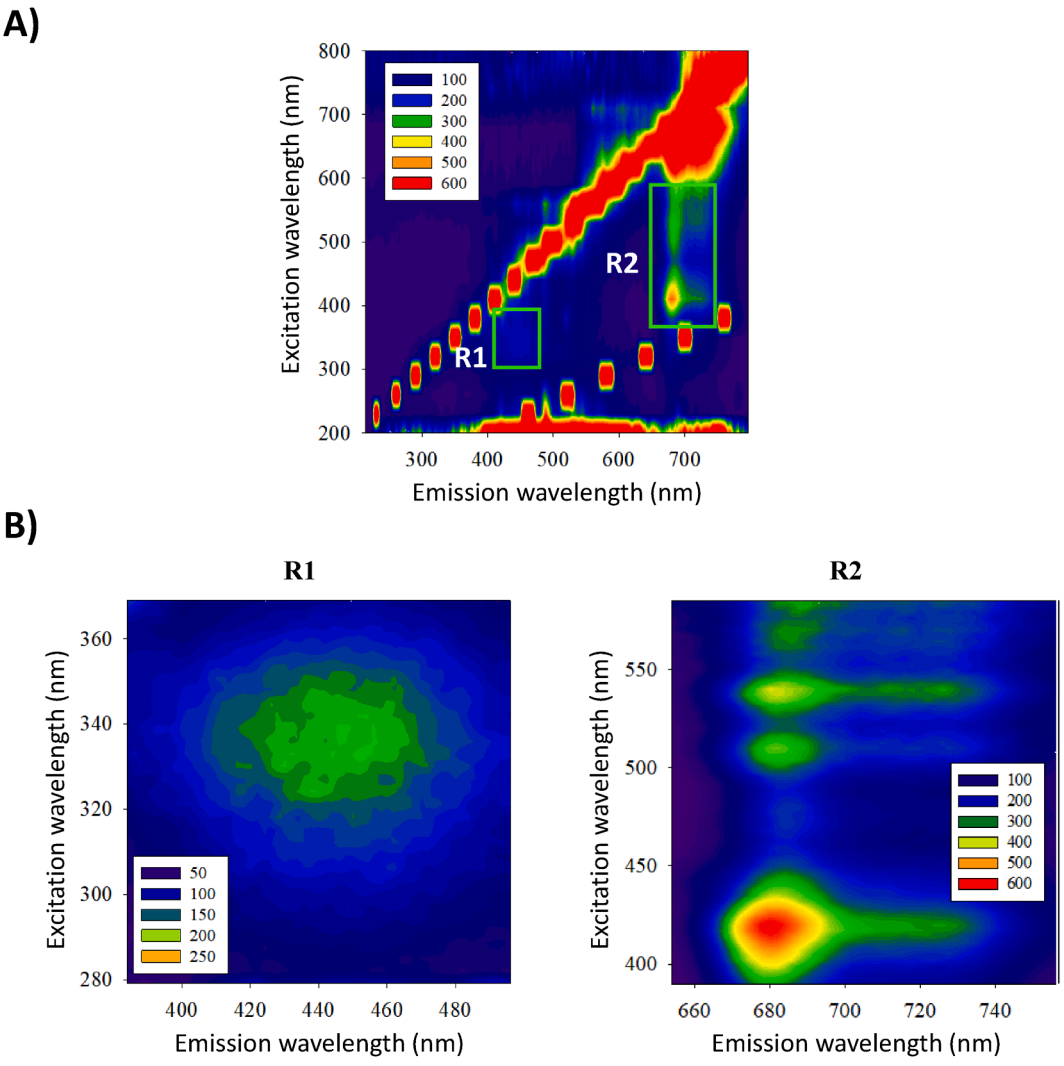

Fig. 1. A) Full EEMs fluorescence of grapevine leaves. B) Spectral region R1 and spectral region R2.

through the equations present in [21]. Total chlorophyll content was calculated as the sum of the concentration of chlorophyll *a* and b.

3. Results and discussion

3.1. Fluorescence regions

In order to obtain a complete fluorescence information, a full EEM was recorded in the range λ_{exc} 200–800/ λ_{em} 214–800 and using the conditions indicated at section 2.2. Fig. 1 shows the full EEM as contour maps of a single sample (Fig. 1A) and two main fluorescence emission areas (Fig. 1B) can be distinguished. The first fluorescence spectral region (R1) has excitation wavelengths from 250 to 380 nm and emission wavelengths from 390 to 500 nm. The second fluorescence region (R2) has high fluorescence signals at emission wavelengths between 650 and 760 nm, with excitation between 390 and 600 nm. No differences in the EEMs physiognomy have been observed between samples of the different geographical regions.

According to the bibliography, in the spectral region R1 several universal cellular fluorophores can emit, namely, nicotinamide, flavin coenzymes or folic acid, and secondary metabolites such as phenolics, alkaloids and terpenoids compounds. In the second spectral region, the fluorescence is only emitted by chlorophylls and pheophytins [22].

With the aim to obtain the maximum sensitivity of each spectral region, both regions were selected and registered separately for further studies. Fig. 1 also shows these two matrices in detail.

3.2. Geographical origin discrimination

The possibility of differentiating between grapevine leaves from different geographic regions through EEMs was analyzed. First, an exploratory analysis was performed through the application of PARAFAC algorithm.

All the data were arranged in two cubic structures (one for each spectral region). The first one with dimensions of 64 × 57 × 40 (samples × number of emission wavelengths × number of excitation wavelengths) and the second one with dimensions of 64 × 52 × 71 (samples × number of emission wavelengths × number of excitation wavelengths). These 3D arrays were analysed independently, and they were decomposed by PARAFAC, using in all cases non-negativity constraints. Different number of components were assayed, and the optimal number was selected based on the largest tested value for which the core consistency is higher than 50% (core consistency diagnostic criterion). For spectral region R1, the optimal number of components was two, while for spectral region R2 the optimal number was three. These results are in accordance with the residual analysis and the physiognomy of the loadings.

The excitation and emission PARAFAC loadings for the two fluorescence regions are shown in Fig. 2. In spectral region R1, the first component presents the maximum excitation wavelength placed at 342 nm and an emission maximum at 430 nm, and this component could not be assigned to a concrete fluorophore. The second component shows the maximum intensity at λex/em = 333/464 nm. This component can be related with chlorogenic acid, being its presence in plant cells already mentioned [23].

Regarding to spectral region R2, the first component shows a very clear and defined shape with an emission maximum at 680 nm, and three excitation maxima at 417, 507 and 537 nm. This component can be assigned to pheophytin [24]. Regarding the second component, it presents several excitation maxima and an emission maximum placed at 732 nm. Finally, the third component shows an emission maximum at 692 nm and an excitation profile with two maxima at 423 and 441 nm and another one at higher wavelengths, at 543 nm. These components could be associated with the chlorophyll *b* and a, respectively [22].

After the analysis of the loadings, the scores values corresponding to each PARAFAC component were plotted against the others (Fig. 3), to explore if the fluorescence spectroscopy data can be useful to discriminate between grapevine leaves from different geographical regions. As can be seen in the Fig. 3, the two principal components in R1 were able to differentiate between the different geographical regions. In fact, the scores of the second component corresponding to samples from Quinta da Leda were higher than those corresponding to Quinta dos Carvalhais. However, in the second spectral region, as can be seen in the tridimensional plot of the scores of the three first PARAFAC components, samples were more overlapped than in the first spectral region, being more difficult to stablish two different groups.

Given the good results obtained through the exploratory analysis by PARAFAC when considering spectral region R1, it was decided to build a classification model using PARAFAC-LDA. In this sense, LDA was applied to the scores obtained in the first two principal components of PARAFAC considering only spectral region R1. The training set was used for data modeling and internal validation with the full cross-validation procedure. This set consisted of 44 samples randomly selected, 22 of each geographic origin. For the external validation, a test set constituted by the remaining 20 samples (10 samples of each geographical origin) was used. Table 1 presents the obtained results of the cross validation and validation, expressed as confusion matrices. As can be seen, almost all the samples were well classified, with a percentage of correct predictions of 93.1% for cross validation and the 95% for validation. In fact, only one sample of Quinta dos Carvalhais was misclassified as belonging to Quinta da Leda in the validation set.

These results suggest that fluorescence spectroscopy combined with PARAFAC-LDA is a powerful tool for the discrimination between grapevine leaves from different geographical origins.

3.3. Qualitative study about the vegetative stage

A qualitative study about the vegetative cycle stage was also performed. The vegetative cycle is a process that takes place in the vineyard each year and comprises all the morphological and biological changes. The occurrence and duration of these changes are influenced by climatic factors [25].

As above mentioned, samples were collected during the ripening period at four different months, because it is known that leaf composition varies significantly during this period which is then reflected in the fluorescent characteristics of grapevine leaves.

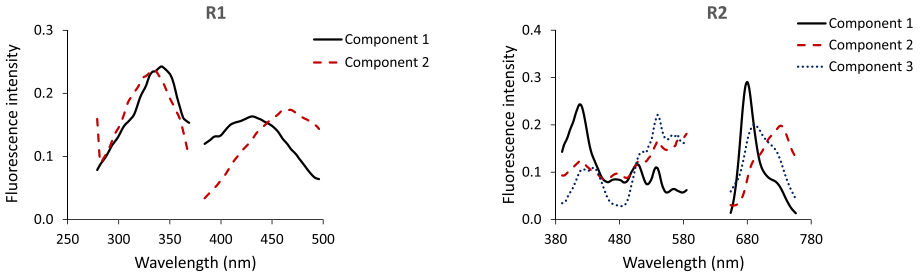

Fig. 2. Excitation and emission PARAFAC loadings for the overall set of samples in spectral regions R1 and R2.

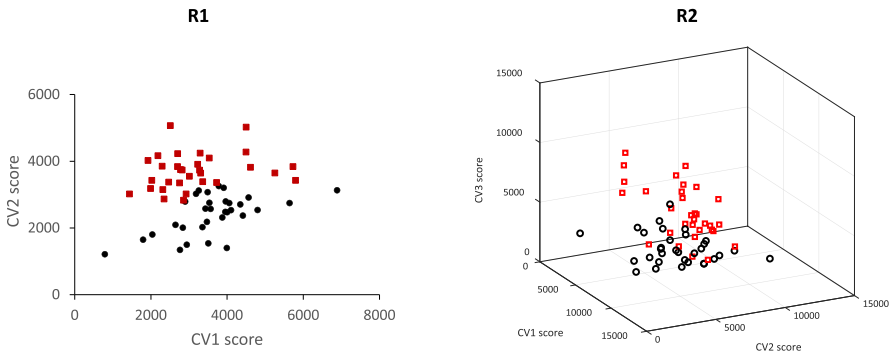

Fig. 3. PARAFAC score plots in spectral region R1 and R2 for grapevines discrimination according to the geographical locations. Quinta dos Carvalhais (black) and Quinta da Leda (red).

Table 1
Confusion matrix obtained through PARAFAC-LDA model for geographical classification of grapevine leaves using the fluorescence region R1. Values are expressed in number of samples.

Real geographical region	Predicted geographical region			
	Cross validation		Validation	
	Quinta dos Carvalhais	Quinta da Leda	Quinta dos Carvalhais	Quinta da Leda
Quinta dos Carvalhais	**20**	2	**9**	1
Quinta da Leda	1	**21**	0	**10**

Diagonal bold contains the number of correct assignments.

Considering the differences found between samples from the two geographical regions, samples were divided according to each geographical region separately. Therefore, each data set was composed by 32 EEMs. When PARAFAC was applied to each this data set, the number of components and the loading profiles were similar for both data sets and their physiognomy were also similar to the results obtained when considering all samples.

Moreover, for each geographical region, the samples loadings of the principal components were analyzed separately with PARAFAC to investigate if they changed along the sampling date. For all the sampling dates, the number of components was the same and the loading profiles did not differ from the ones when all the samples where used.

Fig. 4 shows the evolution of the score values for the principal components along the vegetative stage, considering each spectral region and geographical region separately. The trend in both geographical regions was similar, so the results corresponding to Quinta dos Carvalhais are shown as an example. As can be seen, in spectral region R1 (Fig. 4A), the score values for the first component decrease from June to July and then it remains constant along the time. The score values of the second component has an opposite behavior. First, it remains constant during the first two months and then increases slightly. This behavior is in accordance with the literature where it is indicated that there are not significant differences in the level of chlorogenic acid along the growth stages.

On the other hand, in spectral region R2 (Fig. 4B), the scores of the first and second component increase along the time, while the scores of the third component decrease. This may be connected with the formation cycle of chlorophylls and pheophytins during the vegetative cycle.

3.4. Total polyphenols and chlorophylls quantification

In accordance with the above results, it was studied the possibility of quantifying the amount of total polyphenols and total chlorophylls through the use of spectral regions R1 and R2, respectively.

U-PLS and N-PLS algorithms were tested to stablish quantitative models between the EEMs and the experimental data of total polyphenol and total chlorophylls (sum of chlorophyll *a* and b) content (obtained through the reference procedures).

First, a calibration set with 70% of the samples, was used to build the model. The optimum number of factors was optimized by cross validation and following Haaland and Thomas criterion [19]. The optimum number of latent variables was 4 and 3, for polyphenols and chlorophylls determination, respectively. For both models a good correlation in cross validation was found between the known values from the experimental analysis and the predicted values from the fluorescence data, as can be seen in Table 2. The results were quite similar for both algorithms with coefficients of determination for the cross-validation higher than 0.90 for total polyphenols quantification and higher that 0.80 for total chlorophylls quantification.

Secondly, with the aim of validating the proposed method, the test set was then projected to test the accuracy of the developed models and good results were also obtained for both quantifications. As can be seen in the Table 2, for total polyphenols quantification, the coefficient of determination between the real concentration in validation samples and

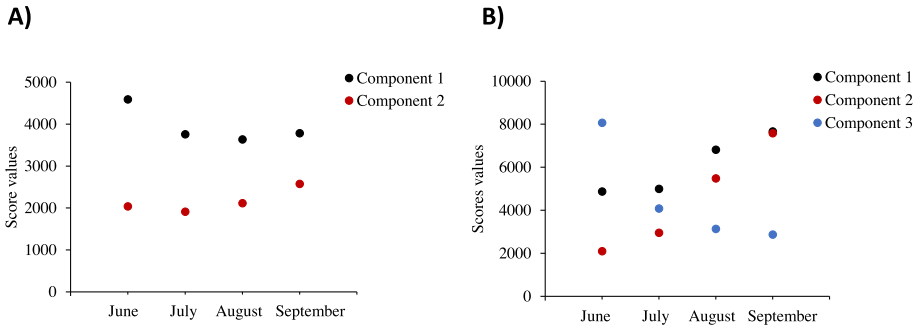

Fig. 4. Evolution of the mean of scores values retrieved by PARAFAC along the sampling data for the spectral region R1 (A) and the spectral region R2 (B).

Table 2
Results obtained for calibration models and test samples with the two algorithms assayed.

Total polyphenols (μg·g^{-1}of sample) (first spectral region)														
	Cross validation								**Validation**					
Algorithm	R2	Slope	Intercept	Anal. Sen.	RMSECV	REP (%)	LOD	LOQ	R2	Slope	Intercept	Anal. Sen.	RMSEP	REP (%)
U-PLS	0.9097	0.99	0.075	0.901	4.62	9.66	4.06	12.6	0.8199	0.79	6.8	0.962	6.97	16.7
N-PLS	0.9055	0.92	3.6	1.25	4.54	9.50	4.30	12.9	0.7631	0.99	0.020	1.33	6.74	16.2
Total chlorophylls (μg·g^{-1} of sample) (second spectral region)														
				Cross validation								**Validation**		
Algorithm	**R^2**	**Slope**	**Intercept**	**Anal. Sen.**	**RMSECV**	**REP (%)**	**LOD**	**LOQ**	**R^2**	**Slope**	**Intercept**	**Anal. Sen.**	**RMSEP**	**REP (%)**
U-PLS	0.8728	0.85	49.5	0.0458	408.8	19.2	29.8	89.6	0.8287	0.82	105	0.0491	383.8	19.6
N-PLS	0.8164	0.81	28.9	0.104	465.0	21.8	52.8	158.4	0.8210	0.75	276	0.111	379.9	19.4

R^2: coefficient of determination; Anal. Sen.: analytical sensitivity; RMSECV: root mean squares error of the cross validation; RMSEP: root mean squares error of prediction; REP: relative error of prediction; LOD: limit of detection; LOQ: limit of quantification.

the predicted concentration by the models, were 0.82 and 0.86 with U-PLS and N-PLS, respectively. For total chlorophylls quantification, the coefficients of determination were 0.83 and 0.82 with U-PLS and N-PLS, respectively.

Table 2 also shows the figures of merit for cross validation and validation. The analytical sensitivity, the root mean square error in both, the cross-validation and prediction, (RMSECV and RMSEP) and the relative error of predictions (REPs) can be considered low for both algorithms. This table also shows the limits of detection (LOD) and limits of quantification (LOQ) through U-PLS and N-PLS. For both algorithms, the limits are lower that the levels of these compounds in grapevine leaves.

Therefore, the obtained results point to the fact that is possible to accurately quantify the total polyphenols and total chlorophylls content in grapevine leaves using fluorescence spectroscopy. Moreover, as the EEMs generated can be considered second order data, this analytical tool is capable of quantifying both these parameters in the presence of uncalibrated species. This is an important improvement regarding the work using NIR and MIR spectroscopy [8] where the data generated is considered as first order data and therefore, susceptible of interference by uncalibrated species.

4. Conclusions

The obtained results in the herein presented work proves the potential of solid state fluorescence in combination with second order algorithms to discriminate grapevine leaves from two different geographical locations and to quantify the total amount of polyphenols and chlorophylls in grapevine leaves along different vegetative cycles. This can hamper grapevine producers to maintain healthy plants (through chlorophyll quantification) in an easy way and enhance the consumption of grapevine leaves (through polyphenol quantification).

The obtained EEMs in combination with PARAFAC showed a high power to discriminate grapevines leaves from different geographical regions. The spectral region connected to the presence of polyphenols (spectral region R1) combined with PARAFAC-LDA allowed the classification of both types of samples with a high percentage of correct predictions (95% for the validation set). Regarding the qualitative analysis using the PARAFAC scores, it was possible to visualize that the obtained scores are capable of reflecting the vegetative cycle that grapevine leaves undergo. This indicates that the EEMs of grapevine leaves retain the chemical changes occurring during the vegetative cycle of leaves.

Moreover, the quantification of important compounds like polyphenols and chlorophylls in grapevines leaves, along the ripening period, was performed for the first time, employing fluorescence spectroscopy with an optical fiber. The EEMs in combination with U-PLS or N-PLS algorithms can be a powerful tool for the quantification of total polyphenol and total chlorophyll content even in the presence of uncalibrated species due to second order advantage. The total polyphenols quantification yielded coefficients of determination for the validation set of 0.82 and 0.76 with U-PLS and N-PLS algorithms, respectively. For total chlorophylls quantification, coefficients of determination for the validation set of 0.83 and 0.82 with U-PLS and N-PLS, respectively, were obtained. The obtained results attest the suitability of the proposed method and demonstrates that both algorithms yielded similar results.

It is worth mentioning that the proposed method avoided the use of chemical reagents and the results were obtained using a single instrument.

CRediT authorship contribution statement

Elísabet Martín-Tornero: Conceptualization, Data curation, Methodology, Formal analysis, Writing – original draft. **Isabel Durán Martín-Merás:** Conceptualization, Methodology, Funding acquisition, Writing – review & editing. **Anunciación Espinosa Mansilla:** Conceptualization, Methodology, Writing – original draft. **João Almeida Lopes:** Funding acquisition, Project administration, Writing – review & editing. **Ricardo Nuno Mendes de Jorge Páscoa:** Conceptualization, Data curation, Methodology, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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