



Determination of phenolic compounds of grape skins during ripening by NIR spectroscopy

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ABSTRACT

The potential of near infrared spectroscopy (NIRS) to determine the content of phenolic compounds in red grapes has been evaluated. The near infrared spectra of intact grapes and grape skins throughout maturity were recorded using a fibre-optic probe and a *transport quartz cup*, respectively. Reference values of phenolic compounds were obtained by HPLC-DAD-MS. Modified Partial Least Squares (MPLS) regression was used to develop the quantitative models for flavanols, flavonols, phenolic acids, anthocyanins and total phenolic compounds. The procedure reported here seems to have an excellent potential for fast and reasonable cost analysis. The results of this work show that the models developed using NIRS technology together with chemometric tools allow the quantification of total phenolic compounds and the families of main phenolic compounds in grape skins throughout maturation. The validation of these models showed the best results for the determination of flavonols (differences between HPLC and NIRS of 7.8% using grapes and 10.7% using grape skins) in the external validation procedure. Good results in the external validation were also obtained for the determination of total phenolic compounds (differences of 11.7% using grapes and 14.7% using grape skins). The best results were generally obtained recording the spectra directly in intact grapes.

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1. Introduction

The content of the different families of phenolic compounds and the total content of phenolic compounds in grapes provide noticeable information about the different ageing potential of wine (Moreno-Arribas & Polo, 2008). The phenolic composition of grapes has also an important role in the definition of the organoleptic properties of wines. Anthocyanins are direct responsible of the color. Flavanols are particularly relevant for astringency (Kallithraka, Bakker, & Clifford, 1998) and flavonols may have some influence on bitterness (Preys et al., 2006). Furthermore, flavanols, flavonols and phenolic acids provide support to wine color and ensure its stabilization during ageing as they can act as anthocyanin copigments. (Boulton, 2001; Gómez-Míguez, González-Manzano, Escribano-Bailón, Heredia, & Santos-Buelga, 2006).

Global methods have been used to quantify polyphenols, such as the Glories method, PTI, Cromoenos® or the Folin-Ciocalteu index among others (Fragoso, Mestres, Bustó, & Guasch, 2010; Kontoudakis, Esteruelas, Fort, Canals, & Zamora, 2009; OIV, 1990),

but these methods are not able to distinguish between different families of phenolic compounds (i.e. flavanols, flavonols, etc.). To achieve this, the use of the high performance liquid chromatography (HPLC) coupled to diode array detection (DAD) and/or mass spectrometry (MS) is necessary (Cheynier & Rigaud, 1986; Goldberg, Karumanchiri, Tsang, & Soleas, 1998; Goldberg, Tsang, Karumanchiri, & Soleas, 1998; Hong & Wrolstad, 1990; Monagas, Suarez, Gomez-Cordoves, & Bartolome, 2005; Wulf & Nagel, 1978). However these techniques are time-consuming and expensive for monitoring changes in phenolic compounds throughout ripening.

Near infrared reflectance spectroscopy (NIRS) is a non-destructive technique, accurate, fast and that can be employed as a replacement for time-consuming chemical methods. NIRS has been proved to be a powerful analytical tool to determine phenolic compounds and other bioactive compounds in foodstuffs (McGoverin, Weerantanaphan, Downey, & Manley, 2010). Infrared spectroscopy has been used for the prediction of procyanidins in cocoa (Whitacre et al., 2003), for the determination of total polyphenol content in green tea using FT-NIR (Chen, Zhao, Liu, Cai, & Liu, 2008), to evaluate phenolic changes occurring during development of olive fruit using ATR-FTIR (López-Sánchez, Ayora-Cañada, & Molina-Díaz, 2009) and to determine malvidin-3-

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glucoside, tannins and pigmented polymers in wine (Cozzolino, Kwiatkowski et al., 2004; Jensen, Egebo, & Meyer, 2008; Skogerson, Downey, Mazza, & Boulton, 2007). This technology has also been used to measure, gluconic acid, glycerol, soluble solids and pH in grape juice (Versari, Parpinello, Mattioli, & Galassi, 2008) and to determine condensed tannins, anthocyanins and the dry matter in homogenised red grape berries (Cozzolino, Cynkar, Dambergs, Mercurio, & Smith, 2008; Janik, Cozzolino, Dambergs, Cynkar, & Gishen, 2007). Moreover, some studies have also been performed directly in intact grape berries in order to determine total anthocyanins (Cozzolino, Dambergs, Janik, Cynkar, & Gishen, 2006; Cozzolino, Esler et al., 2004), the concentration of extractable anthocyanins at pH equal to 1.0 and 3.2, the concentration of total polyphenols, the concentration of sugars and the density (Kemps, Leon, Best, De Baerdemaeker, & De Ketelaere, 2010) using near infrared spectroscopy.

Due to the different roles that the main families of phenolic compounds could play in the chemical, functional and sensory properties of wine, their individual determination is of interest, especially using a single and rapid method of analysis. The aim of this study is to evaluate the potential of NIRS technology to determine the concentrations of the main phenolic families (flavanols, anthocyanins, flavonols and phenolic acids) and the total of phenolic compounds in grape skins and intact grapes. Furthermore, because of the advantages that fibre-optic probe attachment provides in comparison with the use of classic attachments: faster measures, easier clean up and simpler sample preparation; the use of this probe to record the spectra of intact grapes has also been evaluated. To our knowledge, this is the first time that NIRS has been used for simultaneously determining the main phenolic families and total phenolic compounds using a single spectroscopic method.

2. Material and methods

2.1. Samples

Vitis vinifera L. cv. "Graciano" red grape samples were collected from two different vineyards located in La Rioja (Spain). P.O.D La Rioja is divided into regions according to their agro-climatic characteristics. Vineyard 1 is located in Logroño (Rioja Media) and vineyard 2 is located 40 km away, in Haro (Rioja Alta). The first region is under the influence of Mediterranean climate while Rioja Alta generally is cooler and is under the influence of Atlantic climate.

In order to have a wide range of phenolic compounds concentrations to develop more suitable calibrations, the samples were collected at different developmental stages from veraison (September) to over-ripeness (November) in two different vintages (2008 and 2009). In the case of 2008 vintage seven dates were taken into account for vineyard 1 and eight for vineyard 2. For 2009 vintage the number of dates taken into account was six for vineyard 1 and seven for the vineyard 2. Three groups of 150 berries per vineyard were collected at each date. A total of 84 samples were collected in this study corresponding to 39 samples from vineyard 1 and 45 samples from vineyard 2. The berries were collected from both sides of vines located in different rows within the vineyard. Edge rows and the first two vines in a row were avoided. Berries were collected from the top, middle and bottom of the cluster and were immediately frozen and stored at -20°C until analyses were performed. Firstly, the samples were thawed and tempered at room temperature and the near infrared spectra of the intact grapes were recorded at room temperature which was controlled by an air-conditioning device, grape skins were then separated manually

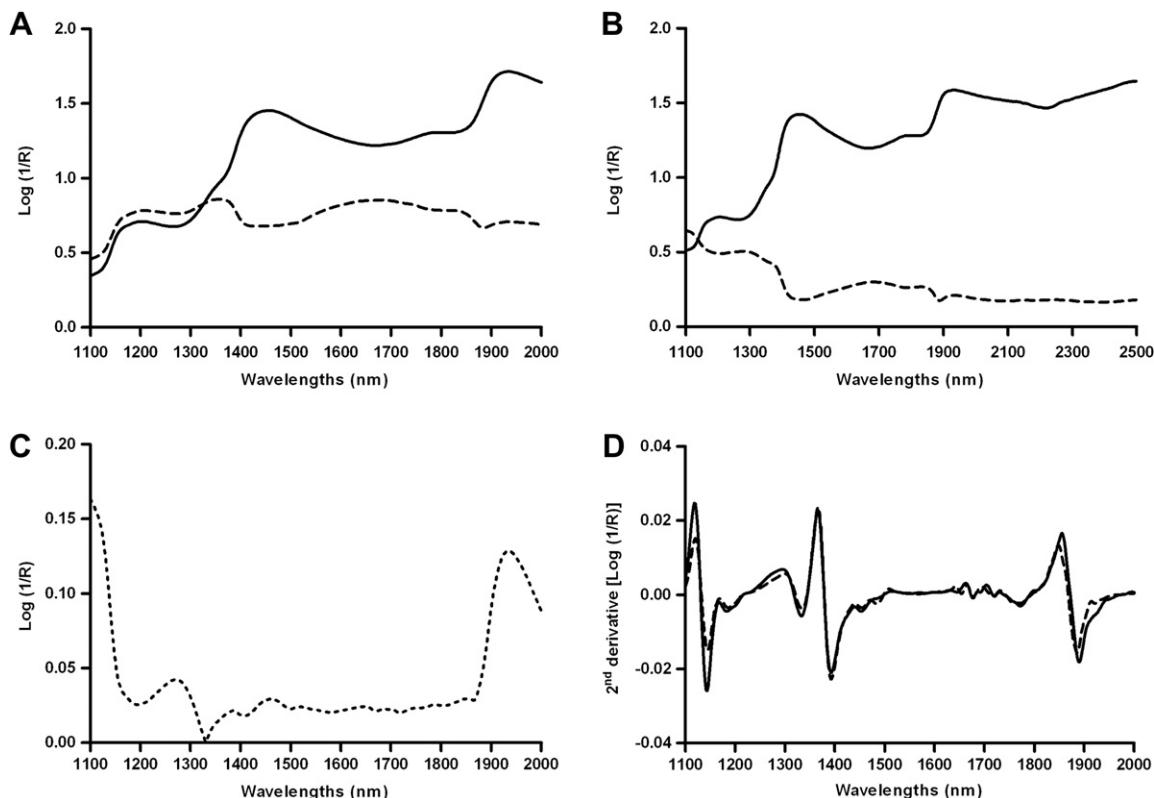


Fig. 1. Average (—) and standard deviation (...) (10 times amplified) spectrum of (A) the whole group of intact grapes (84 samples) in the NIR zone between 1100 and 2000 nm and (B) the whole group of grape skins (84 samples) in the NIR zone between 1100 and 2498 nm. (C) Absolute differences (...) between the mean spectrum of the whole group of intact grapes and the mean spectrum of the whole group of grape skins in the NIR zone between 1100 and 2000 nm. (D) Second derivative of the mean spectrum of grape skins (...) and intact grapes (—) in the NIR zone between 1100 and 2000 nm.

from the whole grapes. Two subsamples were taken from each sample, one for the HPLC analysis and the other one for the near infrared analysis which was also carried out at room temperature.

2.2. Phenolic extraction

Ten grams of grape skins were macerated at 4 °C in methanol (Merck, LiChrosolv®, Darmstadt, Germany) containing 0.1% of 12 M HCl. Methanolic phases were successively pooled, a few milliliters of water were added and the extract was concentrated under vacuum at 30 °C until methanol was removed and finally made up to 100 mL with ultrapure water. The aqueous extract was diluted 1:2 with 0.1 M HCl, filtered through 0.45 µm pore-size filters and directly injected into the chromatographic system to determine the flavonols and anthocyanins. In the case of flavanols and phenolic acids, an aliquot of the aqueous diluted extract was subjected to a clean up procedure using a cationic exchange cartridge (Oasis® MCX). The cartridge was previously conditioned with 2 mL of methanol and 2 mL of Milli-Q water and then the diluted extract (2 mL) was added to the cartridge. Sugars and other polar substances were removed by passing 4 mL of water. The flavonols and phenolic acids were eluted with methanol. The eluate was concentrated under vacuum and redissolved in 500 µL of water. The eluate was injected into the chromatographic system after filtration through a 0.45 µm pore-size filter. All analyses were performed in triplicate.

2.3. HPLC analysis of phenolic compounds

Chromatographic analyses were performed on a Hewlett-Packard 1200 Series HPLC equipped with an auto-injector, quaternary HPLC pump, column heater, diode array detection (DAD) and data treatment station. The HPLC-DAD-MS analyses were carried out in accordance with Ferrer-Gallego et al. (Ferrer-Gallego, García-Marino, Hernández-Hierro, Rivas-Gonzalo, & Escribano-Bailón, 2010) to determine flavanols and phenolic acids. Furthermore, the analyses of anthocyanins and flavonols were carried out in accordance with García-Marino et al. (García-Marino, Hernández-Hierro, Rivas-Gonzalo, & Escribano-Bailón, 2010). Quantification was performed by HPLC-DAD using external calibration curves of purchased standards, unless standards of proanthocyanidins, which were obtained in our laboratory as described by González-Manzano et al., were employed (González-Manzano, Santos-Buelga, Pérez-Alonso, Rivas-Gonzalo, & Escribano-Bailón, 2006). The standard error was generally below 10% so the error and high degree of accuracy of the reference method was considered appropriate to use these data as reference values.

2.4. Near infrared spectroscopy analysis and chemometrics

The eighty four subsamples of grape skins and intact grapes were used to carry out near infrared spectroscopy analysis. A Foss NIRS system 5000 was used. The spectra were recorded at intervals of 2 nm, performing 32 scans for both reference and samples. The fibre-optic probe employs a remote reflectance system and a ceramic plate as reference. The window is of quartz with a 5 × 5 cm surface area, measuring reflectance in the NIR zone over a wavelength range of 1100–2000 nm. The NIR spectra were recorded by direct application of the fibre-optic probe onto the intact grapes. The whole data set was constituted by 84 intact grape samples and 451 data points per sample. Fig. 1 shows the average and standard deviation spectrum of the whole group (84 intact grape samples) in the NIR zone between 1100 and 2000 nm.

The transport quartz cup with a window surface of 4.7 cm × 5.7 cm and an optical pathway of 1.7 cm was used in the 1/2 full mode (i.e. grape skins covering 1/2 of the surface windows) to

record the reflectance spectra of grape skins. In this case, the measurements were made in the NIR zone over a wavelength range of 1100–2498 nm. The whole data set was constituted by 84 grape skin samples and 700 data points per sample. Fig. 1 shows the average and standard deviation spectrum of the whole group (84 grape skin samples) in the NIR zone between 1100 and 2498 nm.

The software used was Win ISI® (v1.50) (Infrasoft International, LLC, Port. Matilda, PA, USA). This software allows not only the spectral acquisition but also the data treatment and the development of the quantitative models. From the three samples of each date, one (33%) was randomly allocated to the validation set and the other two (66%) to the calibration set. Consequently, from the 84 samples of each sample presentation, i.e. intact grapes and grape skins, 56 samples were allocated in the calibration set and the remaining 28 samples were allocated in the validation set.

Prior to the quantitative analysis, principal component analysis (PCA), an unsupervised pattern recognition technique, was used in order to provide information about the latent structure of the spectral data and to identify spectral outliers according to their Mahalanobis distance (H-outliers). These outliers were eliminated at this stage.

The spectral data were pre-treated with Multiplicative Scatter Correction (MSC), Standard Normal Variate (SNV) and Detrend to remove the effects of scattering (Dhanoa, Lister, & Barnes, 1995; Geladi, MacDougall, & Martens, 1985). Moreover, several mathematical treatments were tested in the development of the NIRS calibrations, a, b, c, d, where the first digit is the number of the derivative; the second is the gap over which the derivative is calculated; the third is the number of data points in a running average or smoothing, and the fourth is the second smoothing (Shenk & Westerhaus, 1995). The calibrations were performed by modified partial least squares regression (MPLS), which is often more stable and accurate than the standard PLS algorithm. In MPLS the NIRS residuals at each wavelength obtained after each factor has been calculated were standardised (dividing by the standard deviations of the residuals at each wavelength) before calculating the next factor (Shenk & Westerhaus, 1995). For cross-validation, the calibration set was divided into several groups (6 groups in this case); each group was then validated using a calibration developed with the other samples. In this process another type of outliers (T outliers) was identified which presents high residuals when predicted by the model generated in the cross-validation process. The final calibration models were developed after eliminating these outliers. Finally, validation errors were combined into a standard error of cross-validation (SECV).

3. Results and discussion

3.1. Chemical analysis

Up to 41 phenolic compounds were determined by HPLC-DAD-MS. The phenolic compounds were grouped taking into account their basic structures. The range, mean value and the standard deviation of the concentrations of flavanols, flavonols, anthocyanins and phenolic acids are shown in Table 1.

Table 1

Phenolic compounds content in mg g⁻¹ of grape skins.

	Minimum	Maximum	Mean	SD ^a
Anthocyanins	0.24	11.14	4.41	2.56
Flavonols	0.54	1.75	1.24	0.34
Flavanols	0.41	6.92	3.14	2.08
Phenolic acids	0.10	0.76	0.43	0.17
Total	1.95	18.35	9.23	4.61

^a SD = Standard Deviation (*n* = 84).

Table 2Calibration statistical descriptors^a for the NIRS models in intact grapes (mg g⁻¹ of grape skins).

Spectral pre-treatments	Phenolic compounds	T outliers	N	PLS factors	Mean (mg g ⁻¹)	SD (mg g ⁻¹)	Minimum (mg g ⁻¹)	Maximum (mg g ⁻¹)	SEC (mg g ⁻¹)	RSQ	SECV (mg g ⁻¹)	VC ^b (%)
Std. MSC 2,4,4,1	Anthocyanins	0	56	6	4.41	2.56	0.00	12.10	0.56	0.952	1.10	12.76
SNV 2,8,6,1	Phenolic acids	2	54	5	0.42	0.17	0.00	0.93	0.08	0.747	0.11	20.25
SNV 2,8,6,1	Flavanols	4	52	7	3.11	2.04	0.00	9.23	0.38	0.966	0.59	12.08
None 2,4,4,1	Flavonols	0	56	6	1.24	0.34	0.23	2.26	0.09	0.933	0.16	7.01
SNV 2,4,4,1	Total	2	54	6	9.17	4.69	0.00	23.23	0.63	0.982	1.33	6.87

^a N: number of samples; SD: standard deviation; SEC: standard error of calibration; RSQ: coefficient of determination; SECV: standard error of cross-validation.^b VC (%)=(SEC/Mean)·100.**Table 3**Calibration statistical descriptors^a for the NIRS models in grape skins (mg g⁻¹ of grape skins).

Spectral pre-treatments	Phenolic compounds	T outliers	N	PLS factors	Mean (mg g ⁻¹)	SD (mg g ⁻¹)	Minimum (mg g ⁻¹)	Maximum (mg g ⁻¹)	SEC (mg g ⁻¹)	RSQ	SECV (mg g ⁻¹)	VC ^b (%)
None 1,4,4,1	Anthocyanins	2	54	7	4.17	2.24	0.00	10.90	0.39	0.970	0.66	9.33
Detrend 2,4,4,1	Phenolic acids	1	55	6	0.42	0.16	0.00	0.91	0.04	0.941	0.12	9.55
Std. MSC 2,4,4,1	Flavanols	2	54	7	3.14	2.12	0.00	9.50	0.17	0.994	0.68	5.38
SNV 2,4,4,1	Flavonols	3	53	7	1.28	0.31	0.36	2.20	0.03	0.993	0.11	2.07
SNV 2,4,4,1	Total	0	56	5	9.23	4.61	0.00	23.06	0.65	0.980	1.34	7.07

^a N: number of samples; SD: standard deviation; SEC: standard error of calibration; RSQ: coefficient of determination; SECV: standard error of cross-validation.^b VC (%)=(SEC/Mean)·100.

3.2. NIRS analysis

A spectral pre-treatment (SNV 2,4,4,1) was applied to NIR raw data of samples from the calibration set and then PCA was carried out. This method provides not only information related to the spectral outliers and distribution of the samples in the created space, but it is also an important source of knowledge for the creation of cross-validation groups used in the calibration process. The cross-validation groups were created according to the H distance thus the groups presented a homogeneous distribution of samples among groups but heterogeneous within groups according to their H distance. Moreover, this is a useful tool to identify whether unknown samples do not belong to the spectral space ($H > 3$) created by the samples from which the models were developed (Brereton, 2003; Shenk & Westerhaus, 1995). The spectral variability explained in grapes was 97.9% and 9 principal components were required. For the grape skins the spectral variability explained was 97.1% and 14 principal components were required. It is noticeable that in the case of grape skins more principal components were required to explain similar spectral variability. This could be due to the fact that the spectral data matrix of grape skins presented a more complex structure and the manipulation of the sample often introduces a major source of variation. Thus, the use of intact grapes avoids interferences that may be due to the manipulation of the sample. The spectra of the samples were ranked in order of their H (Mahalanobis) distance from the mean spectrum of all the spectra and the $H > 3$ criterion

was applied. H-outliers were not found either in grapes or grape skins.

The calibrations were performed by modified partial least squares regression (MPLS) testing different spectral pre-treatments. The optimum number of PLS terms used for the calibration was determined by cross-validation taking into account 6 groups. Tables 2 and 3 show the statistical parameters of the final calibration equations for the grapes and the grape skins, respectively. These tables show the number of samples used to obtain the calibration equation (N) after eliminating outliers based on the T value ($T > 2.5$). The best of the different mathematical treatments, concentration range and standard deviation are also shown. The performance of the calibration is expressed as the standard error of cross-validation (SECV).

The spectral regions close to 1140 and 1320 nm presented important contributions to the loadings of the models and are mainly related to combination bands of the –OH functional group, symmetric and anti-symmetric stretching. This wavelength region is also related to C–H aromatic second overtones and C–H third overtones (Osborne, Fearn, & Hindle, 1993; Siesler, Ozaky, Kawata, & Heise, 2002). These can be attributed to the chemical structure of the compounds analysed. Fig. 1 shows the standard deviation spectrum of the whole group and the highest deviation was around 1100–1300 nm which was also the most important zone used in the development of the PLS models in both cases (i.e. intact grapes and grape skins). Moreover, differences in the spectra and the second derivative spectra were observed around the O–H bands regarding

Table 4Internal and external validation of quantitative NIRS models in intact grapes (mg g⁻¹ of grape skins).

Phenolic compounds	Internal validation (N ^a samples)					External validation (28 samples)	
	N ^a	RPD ^b	RSQ ^c	SEP ^c (mg g ⁻¹)	SEP (C) ^c (mg g ⁻¹)	Differences (%)	SEP ^c (mg g ⁻¹)
Anthocyanins	56	4.8	0.957	0.53	0.53	43.9	1.09
Phenolic acids	54	2.1	0.766	0.08	0.08	27.3	0.13
Flavanols	52	5.8	0.971	0.35	0.35	27.8	0.72
Flavonols	56	4.2	0.941	0.08	0.08	7.8	0.12
Total	54	7.9	0.984	0.59	0.59	11.7	1.26

^a N: samples used in the internal validation procedure.^b RPD: ratio performance deviation of internal validation (SD/SEP).^c RSQ: coefficient of determination; SEP: standard error of prediction; SEP (C): corrected standard error of prediction.

Table 5Internal and external validations of quantitative NIRS models in grape skins (mg g^{-1} of grape skins).

Phenolic compounds	Internal validation (N ^a samples)					External validation (28 samples)	
	N ^a	RPD ^b	RSQ ^c	SEP ^c (mg g^{-1})	SEP (C) ^c (mg g^{-1})	Differences (%)	SEP ^c (mg g^{-1})
Anthocyanins	54	4.4	0.974	0.36	0.36	17.5	0.81
Phenolic acids	55	6.3	0.947	0.04	0.04	29.5	0.11
Flavanols	54	13.6	0.995	0.16	0.16	31.9	0.68
Flavonols	53	12.7	0.993	0.02	0.03	10.7	0.15
Total	56	7.5	0.982	0.62	0.62	14.7	1.30

^a N: samples used in the internal validation procedure.^b RPD: ratio performance deviation of internal validation (SD/SEP).^c RSQ: coefficient of determination; SEP: standard error of prediction; SEP (C): corrected standard error of prediction.

the presentation form. This confirms a previous studies that showed differences in the same zone using intact grapes, homogenates of grapes and a single grape (Cozzolino et al., 2006).

In order to evaluate NIRS technology models, an internal validation was carried out using samples that belonged to the calibration group after eliminating outliers (in this case only 1 outlier). The SD/SEP ratio or ratio performance deviation (RPD) indicates that the performances of calibrations were adequate. Tables 4 and 5 show other descriptors of the internal validation. Fig. 2 and Fig. 3 show the NIRS predicted values versus HPLC values in intact grapes and grape skins respectively.

An external validation (Tables 4 and 5) was performed to check the robustness of the method using the 28 samples that did not belong to the calibration group. Calibration models obtained were applied and the predicted values were compared with the reference data.

The differences between the HPLC reference method and the NIRS technique in external validation are generally smaller in intact

grapes than in grape skins except in the case of anthocyanins. For both (grape skins and grapes) minor differences between methods were found in the determination of flavonols. Moreover, the content of total polyphenols show similar and good results in both cases.

Although previous studies have been carried out in intact grape berries in order to determine total anthocyanins (Cozzolino et al., 2006; Cozzolino, Esler et al., 2004), the concentration of extractable anthocyanins at pH equal to 1.0 and 3.2, the concentration of total polyphenols, the concentration of sugars and the density (Kemps et al., 2010) using near infrared spectroscopy none of them has performed the determination of the main families of phenolic compounds (i.e. flavanols, anthocyanins, flavonols and phenolic acids). In the case of total polyphenols, Kemps et al. did not achieve satisfactory results because they considered the total ITP index instead of the total polyphenols from the skins (Kemps et al., 2010). Regarding anthocyanins other studies have been performed taking into account different varieties of grapes and production areas. The

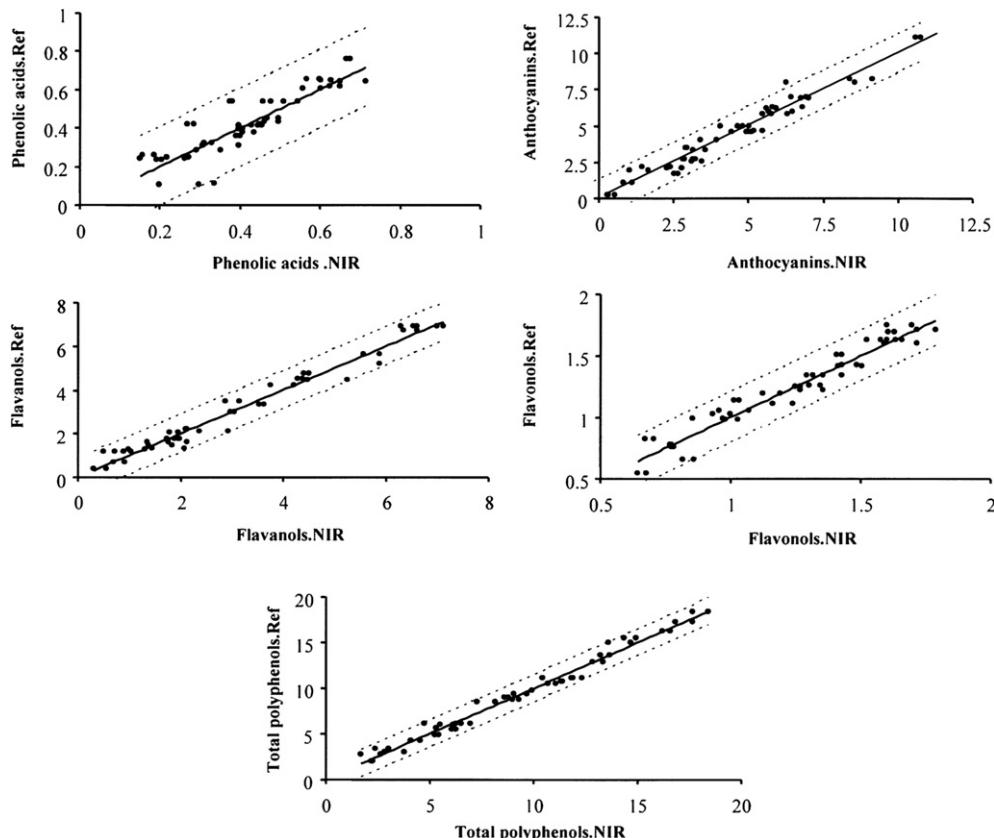


Fig. 2. Comparison of HPLC reference value with the values predicted by the NIRS models in intact grapes (mg g^{-1} of grape skins).

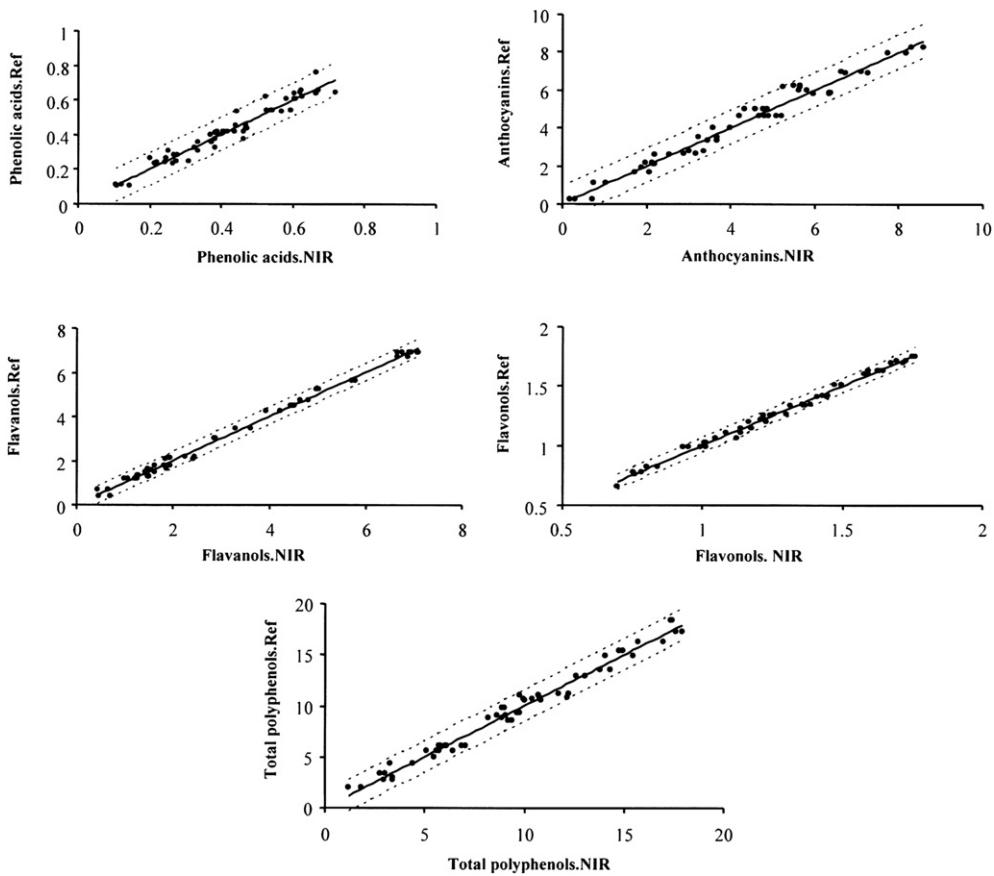


Fig. 3. Comparison of HPLC reference value with the values predicted by the NIRS models in grape skins (mg g^{-1} of grape skins).

SEP values reported for total anthocyanins vary from 0.05 to 0.18 mg g^{-1} of berry (Cozzolino et al., 2006; Cozzolino, Esler et al., 2004). The obtained results (e.g. range and SEP) in our case were comparable to the results obtained in these studies.

4. Conclusions

The results of this work show that the models developed using NIRS technology together with chemometric tools allow the determination, directly in grapes and in grape skins, of the concentrations of total polyphenols and the families of main polyphenols throughout maturation. The best results were generally obtained using a fibre-optic probe and directly recording the spectra of intact grapes, which can be attributable to the practical absence of manipulation of the samples that is needed. The technique reported here is a good option for a rapid quantification of total polyphenols and the families of main polyphenols, the results being comparable with the high-cost and time-consuming analysis methods. The development and application of this technique could become an accurate and efficient tool to aid decision making at harvest time. Nonetheless, a comprehensive study should be made in order to evaluate factors, such as different production areas and grape varieties, in the development of these models.

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