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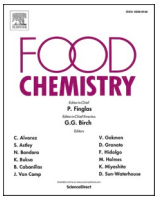

^{1}H NMR and UPLC-HRMS-based metabolomic approach for evaluation of the grape maturity and maceration time of Touriga Nacional wines and their correlation with the chemical stability

Elenilson G. Alves Filho [a], Lorena Mara A. Silva [b,*], Thamires O. Lima [a], Paulo R.V. Ribeiro [b], Cristine S. Vidal [a], Erika S.S. Carvalho [c], Janice I. Druzian [c,1], Aline T.B. Marques [d,*], Kirley M. Canuto [b,*]

[a] Universidade Federal do Ceará, Fortaleza, CE, Brazil
[b] Embrapa Agroindústria Tropical, 60511-110 Fortaleza, CE, Brazil
[c] Universidade Federal da Bahia, Salvador, BA, Brazil
[d] Embrapa Semiárido, 56302-970 Petrolina, PE, Brazil

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ABSTRACT

Touriga Nacional is a well-adapted Portuguese grape variety in São Francisco River Valley (northeastern Brazil). Nevertheless, it has only been indicated to short-term consumption because of the lack of chemical stability, which is attributed to low grape acidity and incomplete phenolic maturity. Therefore, we used Ultra-Performance Liquid Chromatography coupled High-resolution Mass Spectrometry, Nuclear Magnetic Resonance and chemometrics (PCA and PLS-DA) to evaluate the grape maturity and maceration time on chemical composition of wines from two harvest seasons. Moreover, we investigated how these experimental factors could affect their chemical stability. Grapes maturity showed to be the main effect. Overall, phenolic acids and short-chain organic acids were found to be at higher levels in wines produced with unripe grapes from February and shorter maceration time ($p < 0.05$). Proanthocyanidins and other flavonoids were increased in wines macerated for longer time using overripe grapes harvested in July. Furthermore, stable wines were made from overripe grapes, which contained more galacturonic acid.

1. Introduction

Touriga Nacional is a Portuguese variety that provides dark, full-bodied and aromatic wines with purple violet blossoms-like scent (Ali et al., 2011). It is well adapted to the São Francisco River Valley (SFRV) conditions, which is a wine-producing semi-arid tropical region in northeast of Brazil (de Souza Nascimento et al., 2018; dos Santos Lima et al., 2014). In these edaphoclimatic conditions, the grapevine does not come to rest and vegetates continuously throughout the year, yielding two annual harvests. Likewise other SFRV wines, Touriga Nacional have been indicated mainly for fast consumption (first two years after bottling) as they are unstable during the aging. This instability can be identified by the modification of the initial coloration of red wines from ruby red to brown, causing financial losses for wineries and a negative image for the SFRV wines. The lack of chemical stability has been attributed to many factors such as incomplete phenolic maturity and low total acidity of the grapes at the time of harvest (Alves Filho et al., 2019; Castillo-Sánchez et al., 2008).

Maturity corresponds to the stage of development in which several physiological, biochemical and structural changes occur in the grape berry. These changes arise from the synthesis, degradation or translocation of compounds such as sugars, anthocyanins, tannins, organic acids, among others, being influenced mainly by the physiological age of the tissues, environmental factors and viticultural management (Jackson, 2008). For grapes intended for winemaking, the harvest is expected to be carried out only after reaching the technological maturity (TM), which is determined by the ratio between the sugar content and titratable acidity, the aromatic maturity and the phenolic maturity. However, due to the high temperature and incidence of solar radiation on the grapevine in the SFRV, the grape reaches early the ideal soluble solids

* Corresponding authors.
E-mail addresses: lorena.mara@embrapa.br (L.M.A. Silva), aline.biasoto@embrapa.br (A.T.B. Marques), kirley.canuto@embrapa.br (K.M. Canuto).
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content for the desirable alcohol content, while the organic acids present in the berry decrease more rapidly, speeding up the technological maturity. The grape can be harvested before phenolic ripening. As a consequence, in addition to the low acidity, the wine have less color intensity, since the anthocyanins that accumulate during the ripening of the grape will be in lower concentration, affecting its stability (Cadot et al., 2012).

One of the ways to minimize the aforementioned inconveniences has been through maceration. Maceration is a step of great importance in the quality of red wines, as it may influence on the color intensity and color stability, besides other sensory attributes such as flavor and structure (Gómez-Míguez & Heredia, 2004; Gordillo et al., 2016; Sacchi et al., 2005). In traditional winemaking, anthocyanins and other phenolic compounds are extracted from the grapes and solubilize in the wine must at this stage, which occurs concurrently with the alcoholic fermentation process (Busse-Valverde et al., 2012; Petropulos et al., 2014). Phenolic compounds are one of the main chemical substances responsible for the sensory characteristics of red wines, such as color, astringency and bitterness (Garrido & Borges, 2013; Jackson, 2008). In general, the extension of the maceration time improves the stability of wines obtained from grapes that are not sufficiently ripe, since anthocyanins and tannins can condense, forming complexes that enhance the color, in addition to being more stable than free anthocyanin (Cadot et al., 2012).

In this context, we used a non-targeted metabolomic approach by Ultra-Performance Liquid Chromatography coupled to Mass Spectrometry (UPLC-HRMS) and Nuclear Magnetic Resonance (NMR) spectroscopy to assess the influence of the maturation stage and the maceration time on the chemical composition of Touriga Nacional wine produced in the SFRV at two harvest times. Additionally, we investigated how these factors could affect its chemical stability of the wines. Our work is the first metabolomic study and evaluation of the maceration time for this wine variety.

2. Materials and methods

2.1. Analytical standards and reagents

Ultrapure water was obtained from Milli-Q water purification system (Millipore, Bedford, MA, USA). Formic acid, acetonitrile, and methanol solvents were LC-MS grade (Merck, Darmstadt, Germany). Astilbin (98%), gallic acid (98%), caffeic acid (98%), citric acid (99%), ferulic acid (99%), galacturonic acid (97%), kaempferol (97%), myricetin (96%), quercetin (95%), quercetin 3-O-glucoside (90%) were purchased from Sigma Aldrich (St Louis, USA), while procyanidin B dimer (epicatechin-4β → 8-epicatechin, 90%) from Extrasynthese (Genay, France). Potassium metabisulfite was from Synth (São Paulo, SP, Brazil). Pectinolytic enzyme was Pectozim Rouge (Garibaldi, RS, Brazil). Deuterated water (99.9%) and sodium-3-trimethylsilylpropionate (TMSP-d_4, 98%) were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). NaH_2PO_4 PA was from Vetec (Duque de Caxias, RJ, Brazil). PTFE syringe filters (0.22 μm) were purchased from Simplepure (Plano, TX, USA).

2.2. Agronomic experiment and winemaking

The Touriga Nacional grapes were harvested from the Embrapa Semiárido experimental area installed in a commercial vineyard (9° 2′ S, 40° 11′ W, 365,5 m, Lagoa Grande, Pernambuco, Brazil) in two different seasons: February 2017 and July 2017. This plantation was composed of 360 plants. The vines were grafted under the rootstock 'Paulsen 1103′ and conducted in a trellis system with 1.5 m spacing between plants and irrigated by drip. For the wine production, the grapes were harvested in three different stages: before, at and after technological maturity (TM), corresponding to unripe, ripe and overripe berries, respectively. The three harvests occurred during consecutive weeks in February 2017 and July 2017, at intervals of six or seven days, which corresponded to 106 and 112 days after pruning (DAP) for grapes at the unripe stage of maturation (21.6 and 22.5 °Brix), 113 and 119 DAP for grapes at the ripe stage (23.4 and 23.8 °Brix), and 119 and 125 DAP for grapes at the overripe stage (24.5 and 24.6 °Brix), respectively.

The winemaking was performed at three different maceration times during the alcoholic fermentation (AF): 7, 14 and 21 days of maceration. The three maceration times were chosen from previous works on tropical red wines, which led to significant changes in coloring, profile of volatile and phenolic compounds, and antioxidant activity of the product (Alencar et al., 2018; Barbará et al., 2020). This process was carried out at Enology Laboratory of Embrapa Semiárido (Petrolina, Pernambuco, Brazil), in triplicate, according to a traditional method (Blouin & Peynaud, 2012). In each vintage, eight combinations of experimental treatments were provided, corresponding to the different stages of grapes maturation and lengths of maceration, as described in Supplementary Material (Table 1SI). Wines from unripe grapes and 21 days of maceration were not produced, since their high astringency would make them sensorially unacceptable for consumption.

The Touriga Nacional grapes (270 kg/ maturation stage) were stored in a cold chamber at 10 ± 2 °C for 24 h and then were destemmed and pressed in a pneumatic press to yield the wine musts. The musts were transferred to glass carboys (20 L) closed with cylindrical airlock glass valves. Next, potassium metabisulfite at 0.10 g/L and the pectinolytic enzyme Pectozim Rouge at 0.08 g/L were added to the must as preservative. The AF was carried out under controlled temperature (24 ± 2 °C) and started after the addition of commercial yeast *Saccharomyces cerevisiae* var. bayanus (0.20 g/L) (Maurivin PDM™) and the fermentation activator Gesferm Plus™ (0.20 g/L). AF was finished when the density was constant (greater than0.98) and the residual sugars concentration of the wines was confirmed to be below 2 g/L (lasting up to 21 days). Malolactic fermentation was carried out naturally at a controlled temperature (18 °C ± 1). The end of the fermentation was confirmed by assessing the absence of malic acid through paper chromatography (Ribéreau-Gayon et al., 2021). The wines were stabilized by cold stabilization (for 10 days at 0 °C) and using 0.4 g/L of Stabigum AEB Group™ (mixture of gum arabic and metatartaric acid). Before bottling, the content of free sulfur dioxide was corrected with potassium metabissulfite to 50 mg/L. The total wine volume was 160 L/vintage, generating around 107 bottles of wine. The analyses were conducted after 30 days of the bottling. Details about the experimental design are described in Table 1SI.

2.3. UPLC-HRMS analysis

The UPLC-HRMS analyses were accomplished according to the method previously described by Alves Filho and coworkers (Alves Filho et al., 2019). The chromatographic separations were performed on an Acquity/Xevo UPLC-ESI-qTOF system (Waters Co., Milford, MA, USA), equipped with an Acquity UPLC BEH C_{18} column (Waters, 150.0 × 2.1 mm × 1.7 μm) at 40 °C. The mobile phase composed of water and acetonitrile, both containing 0.1% of formic acid, ranged from 2 to 95% of acetonitrile in 15 min at a flow of 0.4 mL/min. The MS spectra were acquired in MS^E tandem negative ionization mode between 100 Da and 1180 Da. The samples (1 mL of wine) were filtered and injected in aliquots of 5.0 μL. Three biological replicates of each treatment were run in triplicate. The compounds were tentatively characterized through molecular formula provided by MassLynx 4.1 software from their accurate masses (error < 5 ppm), isotopic patterns (i-fit) and MS fragmentation patterns as well as literature survey on previous occurrence in grapes and wines using Scifinder Scholar database. In addition, compounds were assigned by comparison with reference standards when available. The instrumental drift was monitored by injecting a solution of quercetin standard (1 μg/mL) every ten injections, then checking its retention time and peak area, which were found to be consistent during the entire analysis (RSD = 1.6 % and 7.7 % for 14 injections, respectively).

Samples were injected three times. For all chromatographic peaks, deviations of retention time of ± 0.05 min and exact mass of ± 0.05 Da were defined as the acceptable limits.

2.4. NMR analysis

The NMR analyses were accomplished according to the method previously described by Alves Filho and coworkers in 2019 (Alves Filho et al., 2019). Briefly, the NMR experiments were performed on an Agilent 600-MHz spectrometer (Palo Alto, CA, USA) equipped with a 5 mm inverse detection One Probe™, using the PRESAT pulse sequence for water suppression (δ 4.75). The samples were prepared by solubilizing pellets obtained from 1 mL of vacuum-dried wines in 600 μL of D_2O (99.9%), containing 1.2 mg/mL of TMSP-d_4, and 0.1 M of the buffer NaH_2PO_4. Afterwards, the pH was manually adjusted to 3.0 ± 0.1 on a Mettler Toledo FE-20 pH meter equipped with a microelectrode, using HCl 0.6 M or KOH 0.6 M (depending on th pH). The wine samples were centrifuged during 5 min at 4032 *g* (6000 rpm in a 100 mm rotor, Model 80-2B Centrifuge, Edulab, Curitiba, PR, Brazil), and approximately 0.6 mL of the supernatant was transferred to 5 mm NMR tubes. The temperature was set to 298 K and TMSP-d_4 was used as internal standard (δ 0.0). Three biological replicates of each treatment were analyzed in triplicate by ^{1}H NMR.

The identification of the constituents was performed through 2D NMR (*g*-COSY, *g*-HSQC, and *g*-HMBC). The molecular structures, ^{1}H and ^{13}C chemical shifts, multiplicity, and constant coupling are available in Supplementary Data. The NMR data were compared with an open access database (*www.hmdb.ca*) and literature (Ali et al., 2011; Alves Filho et al., 2019; Godelmann et al., 2013; Laghi et al., 2014; Larsen et al., 2006; Nilsson et al., 2004; Ogrinc et al., 2003; Pereira et al., 2005; Son et al., 2009).

2.5. Chemometric analysis for UPLC-HRMS and NMR data

2.5.1. Influence of harvest season, maturity stage and maceration time

For the UPLC-HRMS data, Base Peak Intensity Processing (BPI)-format chromatograms comprising the region between 0.65 and 7.12 min were obtained and converted to American Standard Code for Information Interchange (ASCII) for numerical matrices construction. The respective matrix with dimensionality of 92,304 data points (144 chromatograms × 641 variables each) were imported by the program PLS Toolbox™ (version 8.6.2 Eigenvector Research Inc., USA) to handle the multivariate data. An unsupervised method by Principal Component Analysis (PCA) and the supervised one by Partial Least Squares – Discriminant Analysis (PLS-DA) were applied for numerical matrix decomposition using the SVD (Singular Value Decomposition) and SIMPLS (Simplified PLS) algorithms, respectively. Before the chemometric analyses, pretreatment for baseline correction (linear fit algorithm), signals smoothing (Savitsky-Golay polynomial filter under order 1), signals alignment by Correlation Optimized Warping (COW, slack 5 and segment length 50), signals normalization considering the total area were applied over the variables, and mean-centering was applied over the samples (Alves Filho et al., 2020).

As developed for the UPLC-HRMS results, the same chemometric software (PLS Toolbox™) and pretreatments (baseline correction, COW alignment, normalization to area and mean-centering) were applied on the ^{1}H NMR dataset. The spectral region between δ 0.85 and 9.3 was selected excluding the suppression region of the non-deuterated water signal (δ 4.76 to 4.91), which resulted in a matrix composed by 144 spectra and 8,485 variables into each spectrum. Initially, PCA algorithm was applied for evaluation of the harvest time, maturation stage and maceration time (data not shown). In addition, in order to enhance the chemical variability among wines based on the grape maturity, the numerical matrix was decomposed by PLS-DA method. Then, pairwise comparisons were performed between unripe grapes (before the technological maturity) and the two other maturity stages (at and after the technological maturity).

For all classification modeling regarding the UPLC-HRMS and ^{1}H NMR analyses, the number of Latent Variables (LV) was selected in accordance with the following statistical parameters: RMSEC (Root Mean Square Error of Calibration); RMSECV (Root Mean Square Error of Cross-Validation); bias and CV bias values; sensitivity and specificity achieved on cross-validation (Alves Filho et al., 2018). The Venetian Blinds method (groups of data split in non-contiguous block) was used for cross-validation of the supervised analyses employing number of data splits equal 10 with 1 sample per thickness blind.

2.5.2. Stored wine stability and its correlation with the UPLC-HRMS and NMR data

The stability of the wines was evaluated by correlation with color parameters and acidity measured immediately after the winemaking (30 days) and 2.5 years later. The wines were kept in amber wine glass bottles and left on the laboratory bench at room temperature to simulate the shelf-life conditions. The pH measurements were accomplished on a Hanna Edge pH meter (Woonsocket, RI, USA). The color measurements were carried out on Genesys 10 s Thermo Fisher Scientific UV–Vis spectrophotometer (Waltham, MA, USA). The color intensity and tonality values of the wines were determined by the absorbances at 420 nm (A_{420}), 520 nm (A_{520}) and 620 nm (A_{620}), as follows: color intensity = $A_{420} + A_{520} + A_{620}$; color tonality = A_{420} / A_{520}. The respective raw data is available in Table 2SI. The parameters variability from the wines stability after stored was statistically certified by analysis of variance (ANOVA) single factor under confidence level of 95% using the Origin™ 9.4 software, by means comparing using the Tukey test and variance homogeneity among the sample groups by Levene test. Wines were classified as stable when both color and pH did not vary significantly during the storage.

Afterwards, a binary classification modeling by PLS-DA was developed considering the ^{1}H NMR and UPLC-HRMS datasets in order to highlight the relevant compounds that contributed to the wine stability. The same chemometrics parameters described in section 2.5.1 were applied for this wine classification based on the stability.

2.6. Quantification of the discriminant compounds

2.6.1. UPLC-HRMS dataset

Compounds with non-overlapped signal that presented significant changes according to chemometric analysis were relatively quantified by measuring their peak areas through MassLynx™ 4.1 software (Waters MS Technologies). The integration process of the partly overlapped peaks was performed by extracting the exact ion (*m/z*) contained within each chromatogram using deconvolution algorithm. The semi-quantitative results were evaluated by ANOVA single factor in order to statistically certify the differences among the means concentrations (significance level of 0.05, Tukey test for means comparison, and Levene test for variance homogeneity).

2.6.2. ^{1}H NMR dataset

The chemometric analysis-based discriminant compounds with non-overlapped signals were quantified through an external reference method, using a standard solution of sucrose (5.0 mg.L^{-1}) to calibrate the spectrometer. In the Eq. (1) is shown the mathematical principle applied for metabolite quantification using the external reference method. The probe file was updated with all the quantitative parameters required for determination of the compound concentrations in the wine samples. The quantitative results were evaluated by ANOVA single factor to statistically certify the differences among the means concentrations (significance level of 0.05, Tukey test for means comparison, and Levene test for variance homogeneity).

$$P_x = (I_x/I_{std}) \times (N_{std}/N_x) \times (M_x/M_{std}) \times (m_{std}/m) \times P_{std} \quad (1)$$

where the concentration of the targeted compound for quantification

(P_x) is calculated using a known concentration of a standard compound as P_{std} (not necessarily the same quantified molecule); I_x and I_{std} being the integrated area of the targeted compound and standard; N_{std} and N_x being the number of nuclei corresponding to the signal from the standard and the targeted compound; M_x and M_{std} being the molecular mass of the targeted compound and standard; m and m_{std} being the weights of the sample and standard (Holzgrabe & Malet-Martino, 2011; Malz & Jancke, 2005).

2.7. Sensory analysis

The sensory analysis procedure was previously approved by the Research Ethics Committee (CAAE 73983717.9.1001.8052), in compliance with Resolution 466/12, of the National Health Council, Brazil. The sensory profiles of Touriga Nacional wines were evaluated by the trained panel using technique based in the Quantitative Descriptive Analysis (QDA®) developed by Stone et al (1974). Firstly, twenty volunteers were screened as described by Biasoto et al (2014) among students and staff members of the Institute of Viticulture and Enology (Petrolina-PE, Brazil). The judges generated a consensual list with 13 sensory descriptors, including their definitions and references for the panel training, using Kelly's Repertory Grid Method (Moskowitz, 1983). In the descriptive ballot for wines evaluation, descriptors were associated with a 9-cm unstructured scale, anchored at the left and right extremes with the terms "none/weak" and "strong", respectively. The terms selected by the panel to characterize the sensory profile of Touriga Nacional wines, including descriptors of appearance (deep ruby color intensity), aroma (aromatic intensity, red fruits, alcoholic and acetic acid), taste/flavor and mouth sensations (gustative persistence, sourness, bitterness, sweetness, red fruit, alcoholic, astringency and body). After the training, a final selection of the panel was carried out, where each panelist evaluated three of the wine samples, in three replications, using the descriptive ballot. Ten judges that showed adequate discriminative power, reproducibility and consensus with the panel for at least 80% of the descriptors were selected to take part in the final panel (Damasio & Costell, 1991). Overall, each panelist evaluated each wine sample in seven replications, using an incomplete balanced block design proposed by Cochran & Cox (1957). The wines elaborated with the grapes harvested in the two seasons (February 2017 and July 2017) were evaluated separately by the same panel one month after the bottling. The biological triplicates of each treatment were mixed. Wine samples (30 mL) were tested at 18 °C, in wine tasting glasses (International Standards Organization - ISO 3591:1977), coded with three-digit numbers and covered with watch glasses. The sensory descriptive data were evaluated by Principal Component Analysis – PCA, carried out using covariance matrix and XLStat software.

3. Results and discussion

The NMR and MS techniques have been used jointly with chemometrics in metabolomic studies for providing comprehensive chemical characterizations of small organic compounds in wines produced under different viticultural conditions (Alañón et al., 2015). Earlier, we had employed a similar approach to evaluate the effect of the harvest season, conduction system and rootstock on white and red wines from SFRV (Alves Filho et al., 2019). Herein, we performed ^{1}H NMR and UPLC-HRMS analysis for evaluating the grape maturity stage and the maceration time on chemical composition of Touriga Nacional wines from SFRV. These results are presented separately according to the analytical technique: UPLC-HRMS (section 3.1), and NMR (section 3.2).

3.1. UPLC-HRMS based metabolic fingerprint

Twenty-six organic compounds were tentatively identified by UPLC-HRMS, from which nineteen were phenolic compounds (9 flavonoids, 5 hydroxycinnamic acids, 4 proanthocyanidins, 1 phenol acid), along with six hydroxy acids and one apocarotenoid (Table 2SI). The Fig. 1SI shows a representative chromatogram with the compounds annotated following their elution order: galacturonic acid, tartaric acid, citric acid, malic acid, gallic acid, glutathionylcaftaric acid, procyanidin B dimer, caftaric acid, dihydrophaseic acid hexoside, procyanidin B tetramer, procyanidin B dimer, procyanidin B trimer, isopropylmalic acid, caffeic acid, ferulic acid, myricetin-*O*-glucoside, tetrahydroxydimethoxyisoflavone hexose isomer, quercetin-*O*-glucoside, quercetin-*O*-glucuronide, astilbin, tetrahydroxydimethoxyisoflavone hexose isomer, isorhamnetin-*O*-hexoside, myricetin, quercetin, caffeic acid derivative and kaempferol.

Phenolic compounds play an important role on the wine sensorial properties, since they are responsible for aroma, color, flavor, bitterness and astringency. Non-volatile phenolic composition of wine depends on genetic and environmental factors (climate, soil, UV radiation exposure), besides viticultural practices (eg. harvest time and maturity stage), and the winemaking process (eg. maceration, fermentation) (Garrido & Borges, 2013; Jackson, 2008). Phenolic compounds are biosynthetically formed from shikimate pathway, yielding phenolic acids, stilbenes, phenylpropanoids (C_6-C_3) among them. Nevertheless, flavonoids and proanthocyanidins are generated through a hybrid biogenetic route combining phenylpropanoids and acetate moieties (via polyketide). These latter ones are provided by a malonyl-CoA unit to yield the aromatic ring A that completes the flavonoid scaffold (C_6-C_3-C_6) (Garrido & Borges, 2013).

Among the nine flavonoids annotated, seven were flavonols, two isoflavones and one flavanonol. No anthocyanin was identified. In contrast to other phenolic compounds, anthocyanins are better detected in positive ESI mode for being positively charged ions. Anthocyanins, main pigment of red wines, are located in the grape skins, accumulating during ripening and reaching their highest level at the maturity (Cadot et al., 2012; Jackson, 2008; Jordao & Correia, 2012). Flavonols are found in grape berry skins and their biosynthesis is sunlight dependent, accumulating after *veraison* (the onset of the ripening of the grapes). Flavonols are masked by anthocyanins, but they contribute to the colour of red wines by co-pigmentation (Cadot et al., 2012). Kaempferol, quercetin and myricetin are the major flavonols (Jackson, 2008). In fact, these compounds along with isorhamnetin were found in free and/or glycosylated forms in our study. Flavanols are spread in seeds, skins and pulp. Also, no flavanol was found in our samples, but they can undergo reactions of condensation or polymerization, thereby they are converted to proanthocyanidins, which are also responsible for astringency. The highest levels of proanthocyanidins occur usually at the onset of ripening (Cadot et al., 2012; Jordao & Correia, 2012). Although the majority of proanthocyanidins in young wines are dimers or trimers (Jackson, 2008), we characterized a procyanidin B tetramer in this study. In agreement with the work of Oliveira et al. (2018), procyanidin B2 dimer was the major proanthocyanidin in Touriga Nacional grapes grown in SFRV (de Oliveira et al., 2018). Lastly, isoflavones and flavanones, such as genistein and astilbin, have been reported in wines; however, as their occurrence is not as common as the other aforementioned flavonoids, their sensorial properties have not been well-established (Garrido & Borges, 2013).

With regard to the hydroxycinnamic acids, we found glutathionylcaftaric acid, caftaric acid, ferulic acid, caffeic acid, and one unknown derivative in Touriga Nacional. These compounds are present in the pulp and skin of the berry, corresponding to the main class of nonflavonoid phenolics in red wines. They may contribute to bitterness, astringency and sourness and, in general, their maximum content is achieved during ripening, then decrease. Hydroxycinnamic acids usually appear esterified with quinic acid, tartaric acid and glucose units (Cadot et al., 2012; Garrido & Borges, 2013).

Initially, an unsupervised modeling by PCA was applied in order to understand the main chemical variability in Touriga Nacional wines according to the harvest seasons (February or July), grape maturity stages (before, at and after the technological maturity), and maceration

time (7, 14 and 21 days of maceration). Then, multivariate classification analyses by PLS-DA were developed in order to emphasize the compounds variation based on the aforementioned experimental variables (harvest seasons, grapes maturity and their maceration time). Fig. 1 illustrates the classification results of the wines, in which the harvest season (July and February) was the strongest factor for the wines discrimination. The influence plot *vs* modeling errors (Fig. 1b) revealed the absence of outliers negatively affecting the classification modeling.

Wines from grapes harvested in February presented the concomitant higher amounts of citric acid (3), two unknown compounds (5, 7), glutathionylcaftaric acid (8), caffeic acid (16), ferulic acid (17), quercetin *O*-glucuronide (21) and caffeic acid derivative (27), while those produced in July exhibited higher amounts of procyanidin B dimer (9), myricetin (25) and quercetin (26). Oliveira and coauthors found remarkable differences between wines from the two harvest seasons, mainly with relation to presence of anthocyanins, flavanols and proanthocyanidins (de Oliveira et al., 2018). Such intra-annual differences occur in SFRV wines because this Brazilian region presents typically lower temperatures and solar radiation as well as higher relative humidity during the first harvest of the year than in the second one (Alves Filho et al., 2019).

Additionally, effects of the grape maturity and wine maceration time on chemical composition of the wines were further investigated for each season separately. However, only wines produced in February presented chemical variation regarding the viticultural practices, therefore just these results will be featured herein. Fig. 2a shows the LV1 × LV2 scores for classificatory evaluation of wines according to the grape maturation. The respective loadings (Fig. 2c) revealed that the wine produced with unripe grapes presented concomitantly high levels of two unknown compounds (5, 7), glutathionylcaftaric acid (8), caffeic acid (16) and ferulic acid (17), whereas wines produced with over-ripe grapes exhibited higher levels of galacturonic acid (1), procyanidin B dimer (9), procyanidin B tetramer (12), procyanidin B dimer (13), tetrahydroxydimethoxyisoflavone hexose isomer (23), and isorhamnetin-*O*-hexoside (24). In addition to corroborate the aforementioned tendencies observed at LV1, the loadings on LV2 axis highlighted higher content of procyanidin B dimer (9) in wines from ripe grapes than those from unripe grapes. The ANOVA results corroborated only the trends observed for the metabolites **1**, **8**, **16** and **17**. Our result suggests that caffeic acid (16) and ferulic acid (17) were converted to caftaric acid (10). Our findings are in agreement with the previous work reported by Cadot et al. (2012), who found that Cabernet franc wines made from riper grapes contained higher tannin levels (Cadot et al., 2012). In addition, Barbará et al. (2019) reported higher levels of some anthocyanins, flavonols and procyanidin A2 in Syrah wine from SFRV produced with grapes harvested with higher degree of maturation (21–23 °Brix) (Barbará et al., 2019). On the other hand, Ali et al. (2011) and Jordão & Correia (2012) studied the changes occurred in Touriga Nacional berries grown in Portugal, and observed a decrease of the levels of proanthocyanidins and hydroxycinnamates, respectively, in Touriga Nacional berries during the last weeks of ripening (Ali et al., 2011; Jordao & Correia, 2012). This divergence can be attributed to the edaphoclimatic conditions of cultivation of the grapes.

Fig. 3 presents the multivariate classificatory results for the Touriga Nacional wine produced under different maceration times. According to loadings analysis, wine produced after 7 days of maceration presented tendency of higher content of citric acid (3), malic acid (4), unknown (5), caffeic acid (16), ferulic acid (17), caffeic acid derivative (27) and kaempferol (28). On the other hand, the wines macerated for 14 and 21 days contained more galacturonic acid (1), procyanidin B dimer (9), procyanidin B tetramer (12), procyanidin B dimer (13), isoflavone hexose isomer (23) and isorhamnetin-*O*-hexoside (24). Barbará et al. (2019) and Alencar et al. (2018) found higher levels of flavan-3-ols, gallic acid, anthocyanins in Syrah wines from SFRV macerated for longer time (more than 20 days) (Alencar et al., 2018; Barbará et al., 2019). Also, Busse-Valverde et al. (2012) reported higher astringency

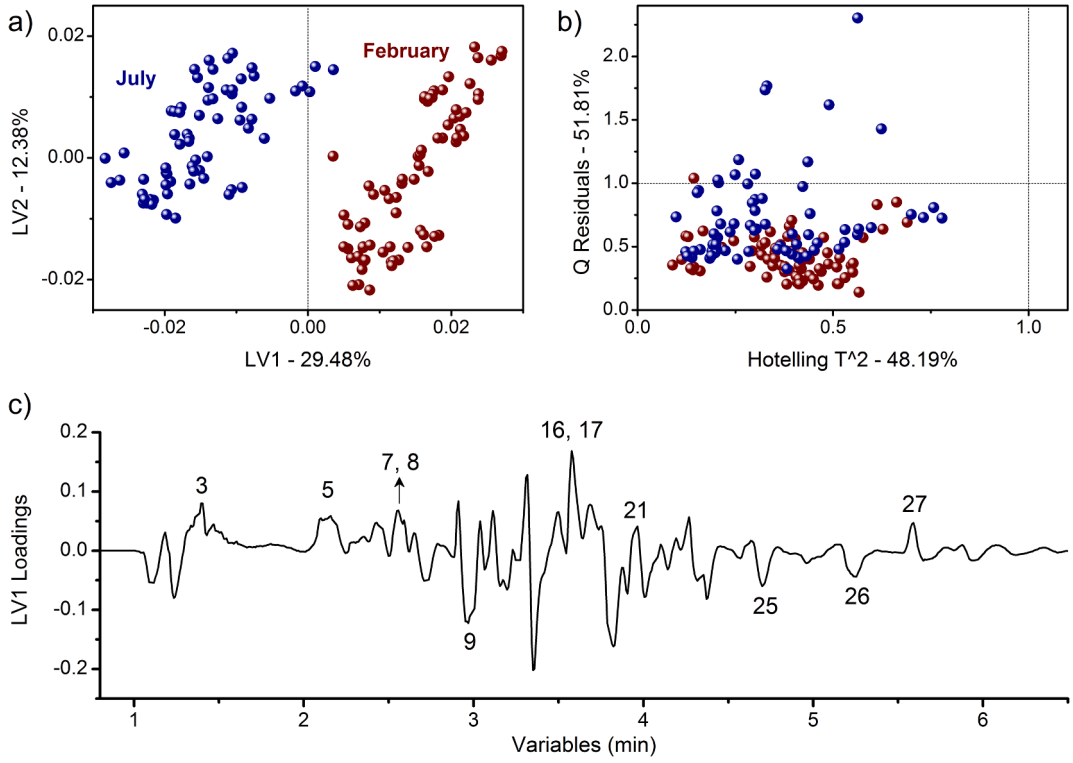

Fig. 1. PLS-DA analysis for the UPLC-HRMS dataset from Touriga Nacional wines produced in July (blue ball) and February (red ball) (a) LV1 × LV2 scores for exploratory evaluation of wines according to the main factor season; b) Influence plot of Hotelling T^2 × Q residuals; c) Loadings plot. Legend: 3 - Citric acid; 5 – Unknown; 7 – Unknown; 8 - Glutathionylcaftaric acid; 9 - Procyanidin B dimer; 16 - Caffeic acid; 17 - Ferulic acid; 21 - Quercetin *O*-glucuronide; 25 - Myricetin; 26 - Quercetin; 27 - Caffeic acid derivative. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

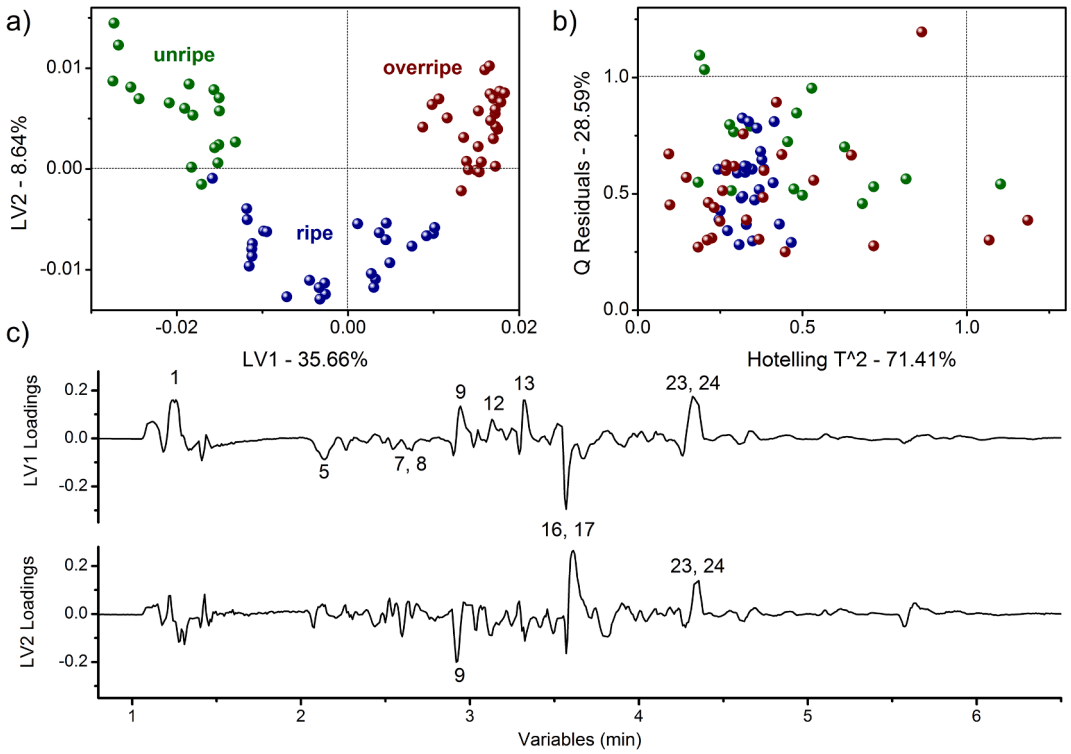

Fig. 2. PLS-DA results for the UPLC-HRMS dataset of the Touriga Nacional wines produced with grapes harvested in February based on grape maturity stage (unripe, ripe, and overripe berries represented as green, blue and red balls, respectively): a) LV1 × LV2 scores for classification of wines according to the maturation stage; b) Influence plot of Hotelling T^2 × Q residuals; c) Loadings plots. Legend: 1 - Galacturonic acid; 5 – Unknown; 7 – Unknown; 8 - Glutathionylcaftaric acid; 9 - Procyanidin B dimer; 12 - Procyanidin B Tetramer; 13 - Procyanidin B dimer; 16 - Caffeic acid; 17 - Ferulic acid; 23 - Tetrahydroxydimethoxyisoflavone hexose isomer; 24 - Isorhamnetin-*O*-hexoside. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

scores to three varietal red wines (Monastrell, Syrah and Cabernet Sauvignon) macerated for 20 days due to increased contents of total proanthocyanidins (Busse-Valverde et al., 2012).

The statistical significance of the metabolites variability was certified by ANOVA (Fig. 5SI) and then, complementing the trends observed by the multivariate statistical analysis: galacturonic acid; citric acid; glutathionylcaftaric acid; procyanidin B dimer; procyanidin B tetramer; caffeic acid; ferulic acid; tetrahydroxydimethoxyisoflavone hexose isomer; quercetin-*O*-glucuronide; isorhamnetin-*O*-hexoside; myricetin; quercetin; caffeic acid derivative; and kaempferol.

3.2. NMR based metabolic fingerprints

The ^{1}H NMR analysis (Fig. 2SI) allowed to identify the twenty-two metabolites in Touriga Nacional wine: the short chain organic acids acetic acid, lactic acid, succinic acid, and tartaric acid; the amino acids alanine, glycine, valine, γ-aminobutyric acid (GABA), leucine, threonine, proline, tyrosine, phenylalanine; the carbohydrates α and β-glucose; the alcohols 2,3-butanediol, ethanol, glycerol; the phenols compounds gallic acid and caffeic acid, along with choline and trigonelline. NMR data from each compound is available on Supplementary Information (Table 3SI).

Due to high complexity of the NMR dataset by the elevate number of identified compounds and experimental procedure, an unsupervised chemometric analyses by PCA method were performed to investigate the variability of Touriga Nacional wines according to the harvest seasons of the grapes (July and February), grape maturity (before the total ripening, at total ripening, and overripe grapes), and wine maceration time (7, 14 and 21 days of maceration). The multivariate exploratory results revealed that the grapes harvest season was the main factor for wines discrimination. Therefore, in order to highlight the variables (compounds) for the wine discrimination, the PLS-DA was further employed to classify the samples in accordance to the previous known groups. Fig. 4 illustrates the multivariate classification results represented by LV1 × LV2 scores (a), influence plot *vs* modeling errors (b), and the loadings plot for samples scores (c).

The PLS-DA results show a trend of separation among the wines based on the harvest season. Wines produced in July, which are located at positive scores on LV1, present concomitantly more 2,3-butanediol, glycerol, lactic acid, succinic acid, tartaric acid, and α and β-glucose. Interestingly, we found earlier that another SFRV red wine (Syrah) contained more 2,3-butanediol, glycerol, succinic acid and tartaric acid in February, even though it was more abundant in lactic acid and proline in July (Alves Filho et al., 2019). This finding indicates that different grape varieties may present variation in their seasonal responses, even when subjected to similar climatological and oenological conditions.

In addition, the chemical variability among wines considering the grape maturation stages as classes was also evaluated by PLS-DA. Fig. 5a illustrates the scores plot of LV1 *vs* LV2, influence plot *vs* modeling errors (b), and the relevant loadings with the most important metabolites for samples discrimination based on wine class (c). The samples scores show that the chemical composition of the wines produced from ripe and overripe grapes (positive LV1 scores) are more similar than the ripe and unripe ones (negative LV1 scores).

PLS-DA analysis revealed higher content of 2,3-butanediol in the wines produced with mature grapes. On the other hand, lactic acid, succinic acid, choline, and tartaric acid were found to be more abundant in wines made with non-mature grapes. Indeed, this result is expected, since the maturation causes a decrease of organic acid, which are either converted to sugars or used as carbon and energy sources for respiration (Conde et al., 2007). Previously, Ali et al. (2011) used ^{1}H NMR and chemometrics to monitor the biochemical changes during the ripening of Touriga Nacional berries. They observed that the unripe grapes (green and *veraison* stages) were characterized by higher levels of phenolics and

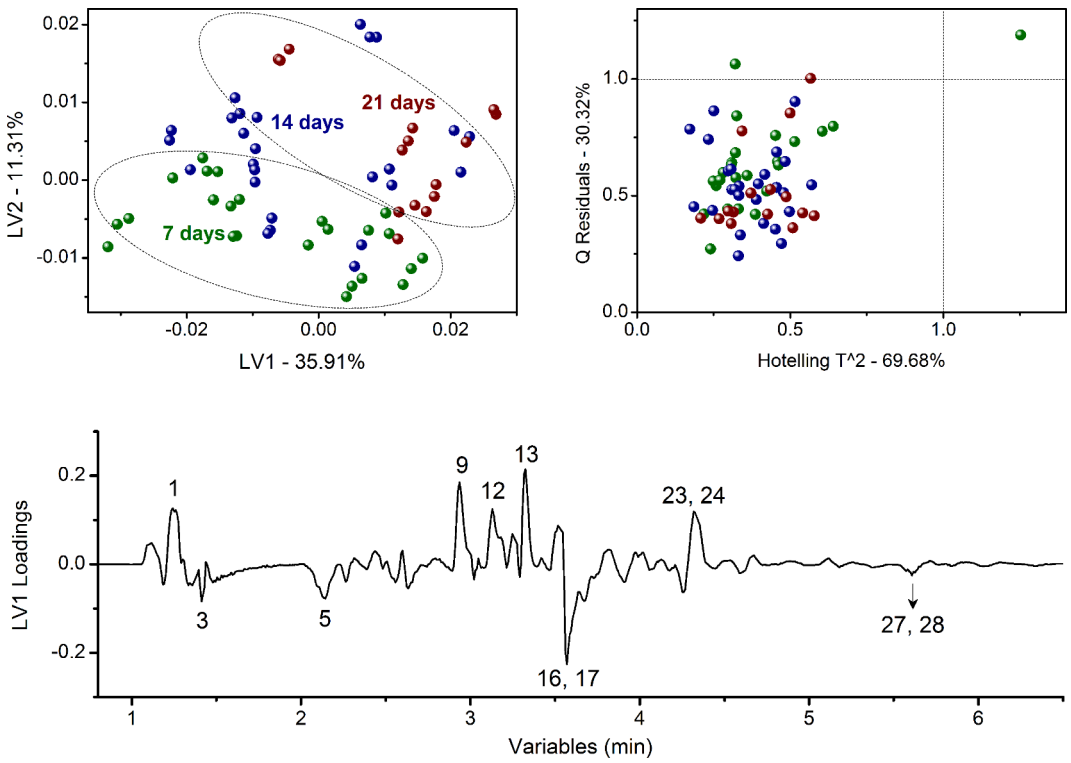

Fig. 3. PLS-DA results for the UPLC-HRMS dataset of the Touriga Nacional wines from grapes harvested in February based on the maceration time (7, 14 and 21 days) represented as green, blue and red balls, respectively): a) LV1 × LV2 scores for classification of wines according to the maturation stage; b) Influence plot of Hotelling T^2 × Q residuals; c) Loadings plots. Legend: 1 - Galacturonic acid; 3 - Citric acid; 5 – Unknown; 9 - Procyanidin B dimer; 12 - Procyanidin B Tetramer; 13 - Procyanidin B dimer; 16 - Caffeic acid; 17 - Ferulic acid; 23 - Isoflavone hexose isomer; 24 - Isorhamnetin-*O*-hexoside; 27 - Caffeic acid derivative; 28 - Kaempferol. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

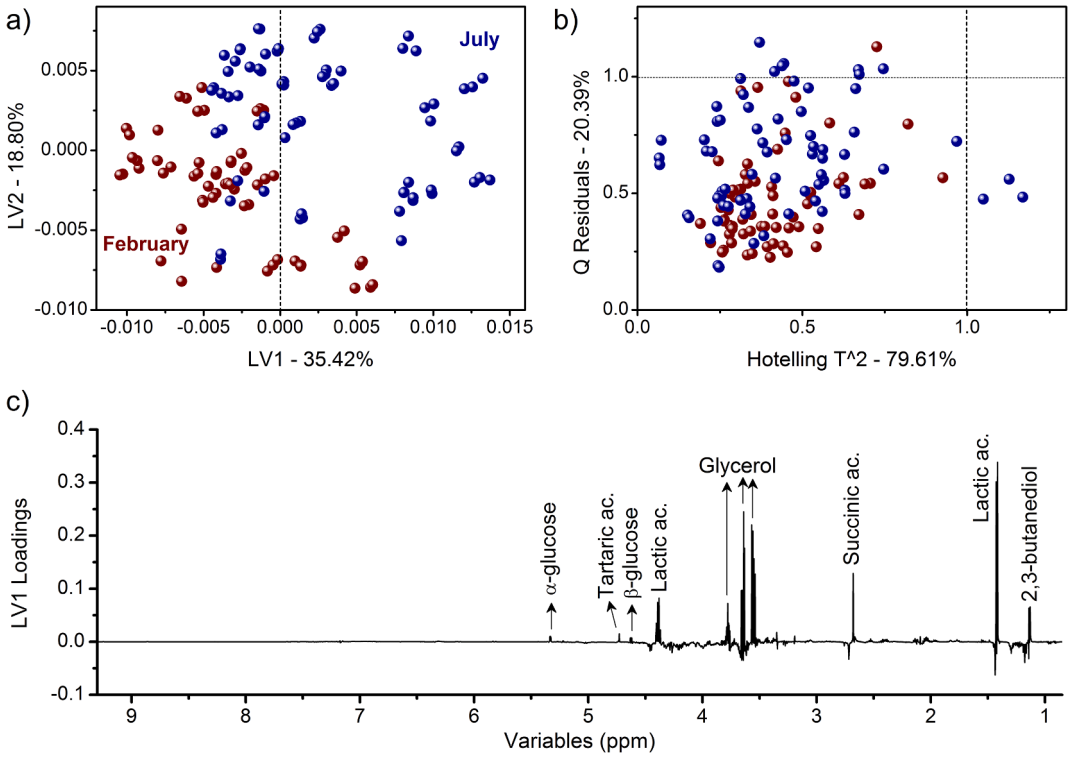

Fig. 4. PLS-DA results for ^{1}H NMR dataset from Touriga Nacional wines according to the harvest season (July- blue ball and February- red ball): a) LV1 × LV2 scores for classification of wines according to the maturation stage; b) Influence plot of Hotelling T^2 × Q residuals; c) loadings plot for LV1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

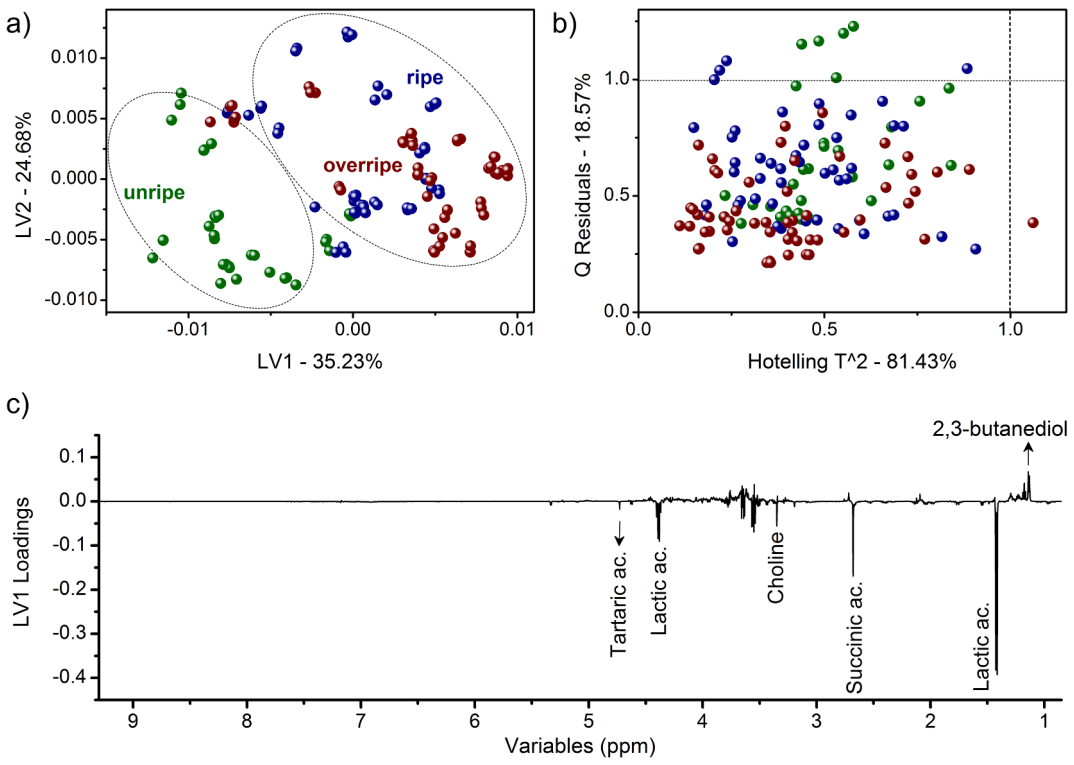

Fig. 5. PLS-DA for ^{1}H NMR dataset from Touriga Nacional wines based on the grape maturity stage (unripe, ripe and overripe berries represented as green, blue and red balls, respectively): a) LV1 × LV2 scores for classification of wines according to the maturation stage; b) Influence plot of Hotelling T^2 × Q residuals; c) Loadings plot for LV1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

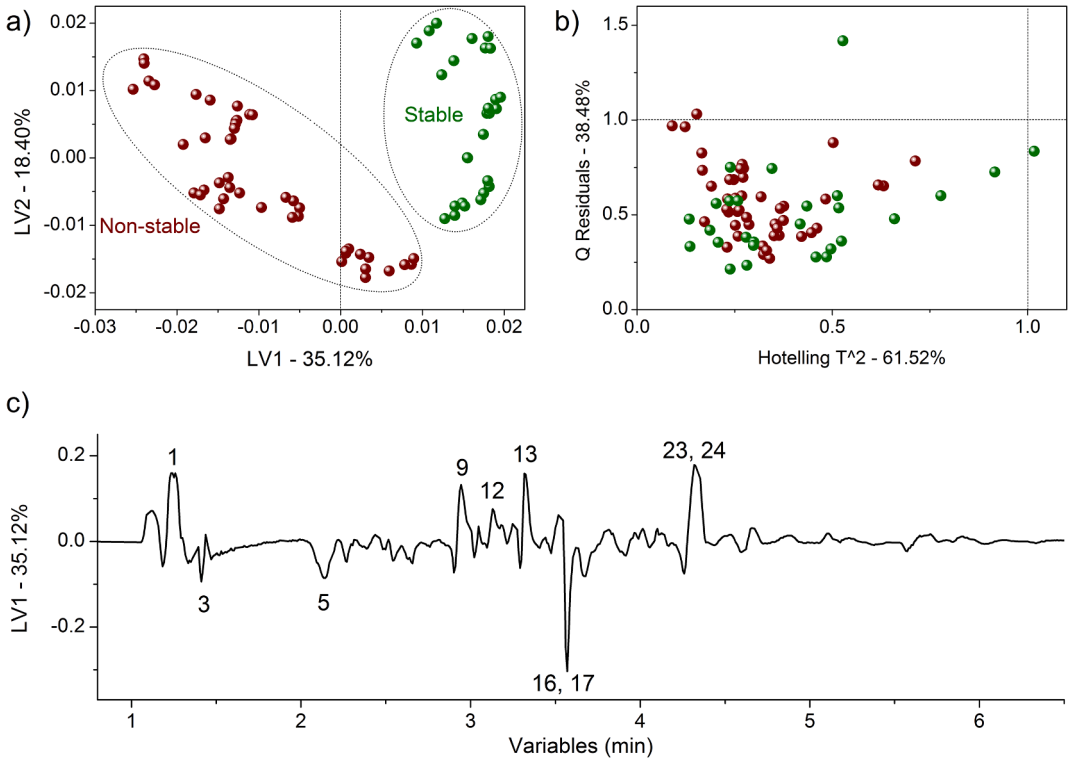

Fig. 6. PLS-DA results for UPLC-HRMS dataset from Touriga wines made with grapes harvested in February and stored for 2.5 years: a) LV1 × LV2 scores for classification of wines according to the stability (stable-green balls and non-stable- red balls); b) Influence plot of Hotelling T^2 × Q residuals; c) Loadings plot. Legend: 1 - Galacturonic acid; 3 - Citric acid; 5 - Unknown; 9 - Procyanidin B dimer; 12 - Procyanidin B Tetramer; 13 - Procyanidin B dimer; 16 - Caffeic acid; 17 - Ferulic acid; 23 - Tetrahydroxydimethoxyisoflavone hexose isomer; 24 - Isorhamnetin-O-hexoside. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

organic acids, whereas the ripe and harvest stages were found with more amounts of amino acids and sugars.

In order to examine deeply the effect of maceration time, six PLS-DA models were performed for the wines from grapes in different maturation stages (unripe, ripe, and overripe) harvested in the two seasons (February and July) (Fig. 3SI). Overall, the maturation did not affect the metabolic fingerprints according to the loadings plots, except for the wines made with unripe grapes harvested in July (Fig. 3bSI) and the wines from ripe grapes produced in February (Fig. 3cSI). The respective loadings (Fig. 4SI) indicate that wines produced in these conditions presented concomitant higher content of 2,3-butanediol, glycerol, lactic acid, succinic acid, gallic acid and α-glucose as macerated for longer time.

Complementing the trends observed in the multivariate statistical analysis, no statistical significance among the concentrations variability of the discriminant compounds was found by the ANOVA results of the ^{1}H *q*NMR data (Fig. 6SI).

3.3. Wines stability under storage

Wines stability was evaluated based on alterations related to the color intensity ($A_{420} + A_{520} + A_{620}$), tonality ($A_{420} / A_{520}$) and pH under storage during 2.5 years. The analysis of variance (ANOVA) of these mentioned parameters revealed that they did not statistically vary in stored wines from overripe. Hence, the grape maturity stage is the key factor to produce more stable wines regarding coloration and acidity.

Afterwards, a binary classification analysis by PLS-DA was developed for the wine stability (stable and non-stable wines), considering the variation of color and pH during the storage. This evaluation was based only on the chemical profile provided by UPLC-HRMS data, since no relevant result was observed for ^{1}H *q*NMR. Fig. 6a illustrates the wines from overripe grapes in green color (stable wines) and those wines from unripe and ripe grapes (non-stable wines) in red. The influence plot *vs* modeling errors showed the absence of outliers negatively affecting the classification modeling (Fig. 6b). According to the loading, the most stable wines contained increased amount of galacturonic acid (1), procyanidin B dimer (9), procyanidin B tetramer (12), procyanidin B dimer (13) tetrahydroxydimethoxyisoflavone hexose isomer (23) and isorhamnetin-*O*-hexoside (24), but less citric acid (3), unknown compounds (5), caffeic acid (16) and ferulic acid (17) than the other samples. Therefore, the compounds 1, 9, 12, 13, 23 and 24 are supposed to be related to the wine stability during storage, once they are more abundant in the stable wine samples. However, only galacturonic acid (1) was found to be significantly higher in the stable wines.

3.4. Wines sensory analysis

The sensory profile of the Touriga Nacional wine from São Francisco River Valley region was impacted by the effect of the harvest season and the grapes maturity stage (Fig. 7SI). According to the harvest season (Fig. 7aSI), the PCA results indicated higher values of aromatic intensity, sourness, alcoholic aroma and flavor, gustative persistence, fruity flavor, and body in wines from February, whereas those wines from July had higher acetic acid aroma at negative scores of PC1 and PC2. Moreover, wines from unripe grapes stood out in the intensity of aroma of red fruits, while the overripe grapes yielded wines with higher deep ruby color intensity, astringency, body, gustative persistence, sweetness, alcoholic and fruity flavors (Fig. 7bSI). Ripe grape wines showed intermediate sensory profile and occupied the central region on the scores plot, therefore, they did not stand out in any of the 13 attributes selected by the sensory panel. Thus, such features appear to be related to tendency of variations in the levels of hydroxycinnamic acids, proanthocyanidins, flavonoids and short-chain organic acids in the aforementioned wines as discussed earlier using UPLC-HRMS and ^{1}H NMR dataset.

4. Conclusion

Our untargeted UPLC-HRMS and ^{1}H NMR approaches with chemometrics tools provided complimentary information for evaluation of the harvest season, grape maturity stage and maceration time on the chemical composition of Touriga Nacional wines. While UPLC-HRMS discriminated the wines for the three aforementioned effects, ^{1}H NMR showed only trend of separation among the samples. The harvest season followed by the grape maturity stage were the most evident effects in both methods. Overall, phenolic acids and short-chain organic acids were found to be at higher levels in wines produced in February with grapes at early maturity stages and shorter maceration time. On the other hand, proanthocyanidins and other flavonoids increased in wines macerated for longer time using ripe and overripe grapes harvested in July. In addition, wines from overripe grapes showed to be more stable, and they were found to contain higher galacturonic acid. Therefore, we look forward that our results can be useful for the improvement of the chemical stability and quality of red wines produced in SFRV region.

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CRediT authorship contribution statement

Elenilson G. Alves Filho: Methodology, Formal analysis, Investigation, Writing – review & editing. **Lorena Mara A. Silva:** Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Thamires O. Lima:** Data curation, Investigation. **Paulo R.V. Ribeiro:** Methodology, Investigation. **Cristine S. Vidal:** Methodology. **Erika S.S. Carvalho:** Methodology, Investigation. **Janice I. Druzian:** Conceptualization, Supervision. **Aline T.B. Marques:** Conceptualization, Supervision, Funding acquisition, Project administration. **Kirley M. Canuto:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition, Project administration.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132359.

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