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Verification of the selectivity of a liquid chromatography method for determination of stilbenes and flavonols in red wines by mass spectrometry

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Abstract Quantification of bioactive phenols, like stilbenes and flavonols (SaF), has been conducted to evaluate the nutraceutical potential of red wines. However, there is still a lack of full validated, fast and accessible liquid chromatography methods offering high selectivity and a simple procedure. We present here the use of a high-resolution mass spectrometer to evaluate the selectivity of a feasible and traditional liquid chromatography technique (HPLC–DAD) to analyze markers of aglycone SaF in red wines. The SaF compounds were tested: *trans*-resveratrol, *trans*-ε-viniferin, quercetin, myricetin, and kaempferol, as well as *trans*-cinnamic acid, one of their precursors. System suitability and validation tests were employed for the selected conditions (octylsilane column, methanol mobile phase, and gradient elution). The validation process ensured the HPLC–DAD method was selective, linear, sensitive, precise, accurate and robust. The method was then applied to red wine samples from the Campanha Gaúcha region, Southern Brazil. The real samples contained different SaF levels, showing that the method is applicable to routine use. Furthermore, this was the first SaF characterization of red wines from the Campanha Gaúcha, contributing to regional and product development.

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Abbreviations

Symbols

t_R	Retention time (min)
V/V	Volume/volume (%)
R_s	Resolution
TF	Asymmetry
k	Capacity factor
N	Theoretical plates
r^2	Coefficient of determination
F_{calc}	Calculated Fisher value
F_{tab}	Fisher table value
n	Number of repetitions
p	Probability
m/z	Mass/charge (Da)

Greek letters

λ_{max}	Maximum ultraviolet absorption wavelength (nm)

Introduction

Biological studies, dealing with the French Paradox, showed that red wines are associated with increased longevity and the prevention of diseases in moderate consumers (Bidlack and Wang 2006; Renaud and De Lorgeril 1992), and that such responses could be mainly attributed to the wine phenol constituents (Flamini et al. 2013; Khan et al. 2013). The wide variety of phenolic compounds found in wines has as their precursor *trans*-cinnamic acid.

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They are derived from the secondary metabolism of grapes and may be classified into non-flavonoid and flavonoid groups (Crozier et al. 2006; Ribéreau-Gayon et al. 2006). Among these classes, the bioactive stilbenes and flavonols (SaF) are the focus of the nutritional aspects of grapes and their derived products, which are some of the major sources of such compounds in the human diet (Bidlack and Wang 2006; Malovaná et al. 2001; Monagas et al. 2005; Pereira et al. 2010; Pérez-Magarino et al. 2008; Rodríguez-Delgado et al. 2002; Silva et al. 2011; Tenore et al. 2011; Villiers et al. 2004; Vitrac et al. 2005; Vrhovsek et al. 2012).

Stilbenes are non-flavonoid molecules, such as *trans*-resveratrol, *trans*-piceid and viniferin, whereas flavonols are from the flavonoid class, such as quercetin, kaempferol and myricetin (Crozier et al. 2006; Ribéreau-Gayon et al. 2006). *Trans*-resveratrol and quercetin, for example, have been extensively evaluated due to their biological activities in the cardiovascular system, brain protection, antioxidative processes, and antitumor and anti-inflammatory actions (Baur et al. 2006; Bidlack and Wang 2006; Flamini et al. 2013; Prior 2006; Tringali 2012). The majority of the grape phenols naturally occur as glycosides, but the wine fermentation processes in acid media usually promote the hydrolysis of the glycoside linkage giving the corresponding aglycone molecules, which are easier to absorb through the gastrointestinal system (Bidlack and Wang 2006; Crespy et al. 1999, 2002; Murota and Terao 2003).

The development of wines containing high levels of SaF may enhance the health benefits already identified in humans (Cotea et al. 2012). It may be substantially supported by a chemical characterization of the SaF biosynthetic pathways, where some SaF markers would be detected in higher doses than their common precursors (e.g., *trans*-cinnamic acid) (Baur et al. 2006; Prior 2006). UPLC (ultra-performance liquid chromatography) coupled to a MS (mass spectrometer) is the most modern technique and, in principle, can create faster and more selective methods than HPLC (high-performance liquid chromatography) coupled to a DAD (diode array detector) (Cielecka-Piontek et al. 2013). Indeed, UPLC-MS should be the first choice for SaF quantification in wines. However, UPLC-MS is not widely available in food laboratories yet, because of the high costs associated with purchasing, and the need for maintenance and skilled personnel. Therefore, it seems clear that the HPLC–DAD may be a suitable analytical option for several laboratories, since it is still the most widespread LC (liquid chromatography) instrumentation worldwide, mainly in wineries or small research centers, where the funding and resources are sometimes restricted. Some previous publications used HPLC–DAD for SaF determination in wines (Adrian et al. 2000; Aznar et al. 2011; Cotea et al. 2012; Malovaná et al. 2001; Pereira et al. 2010; Pérez-Magarino et al. 2008; Villiers et al. 2004; Vitrac et al. 2005). A simple direct injection of the samples for analysis of resveratrol and other SaF has been described in some methods (Adrian et al. 2000; Careri et al. 2004; Castellari et al. 2002; López et al. 2001; Pereira et al. 2010; Revilla and Ryan 2000; Vitrac et al. 2005). Complex sample preparation has been required by other procedures (Baptista et al. 2001; Cotea et al. 2012; Fontana and Bottini 2014; Kerem et al. 2004; Lima et al. 1999; Malovaná et al. 2001; Paulo et al. 2011; Pérez-Magarino et al. 2008; Villiers et al. 2004), but they may introduce analytical errors, be expensive, cause environmental pollution due to chemical waste and be time-consuming.

The evaluation of the selectivity for each chromatographic peak is another important aspect, since it is a crucial validation requirement for such methods (ANVISA 2003; FDA, US Food and Drug Administration 2001; González et al. 2014; ISO International Organisation for Standardisation 2005). Concerning SaF determination in wines, a full exploration of selectivity data is indispensable because the compounds have similar chemical characteristics among themselves and with other organic compounds in the matrix, such as the high levels of red wine pigments (anthocyanins, a positively charged flavonoid class). Interfering compounds may exhibit coelution and, consequently, lead to overestimation of the content, or non-selective detection of, the analytes (González et al. 2014). The DAD detector and, mainly, high-resolution mass spectrometers (e.g., a hybrid quadrupole time-of-flight, or QTOF, instrument) may be able to provide more data through software tools, such as providing the purity index of the chromatographic peaks (ANVISA 2003; FDA US Food and Drug Administration 2001; Kazakevich and Lobrutto 2007). Despite this, selectivity has not been extensively investigated for these analyses when DAD is the detector (Adrian et al. 2000; Aznar et al. 2011; Castellari et al. 2002; Cotea et al. 2012; Gómez-Alonso et al. 2007; Kerem et al. 2004; Lima et al. 1999; López et al. 2001; Malovaná et al. 2001; Pereira et al. 2010; Pérez-Magarino et al. 2008; Rastija et al. 2009; Revilla and Ryan 2000; Villiers et al. 2004; Vitrac et al. 2005). A few selective and validated HPLC–DAD methods were found (Baptista et al. 2001; Careri et al. 2004; Fontana and Bottini 2014), but they did not focus on SaF markers. In the literature, no HPLC–DAD method that combines a short analysis time (below 30 min), simple sample preparation, and full validation with high selectivity has so far been reported. Conditions like these are useful to combine time-reduction with good laboratory practices (Lorrain et al. 2013). Thus, this study aimed to employ QTOF to check the selectivity of a feasible HPLC–DAD method for chemical characterization of the SaF biosynthetic pathways (*trans*-resveratrol, *trans*-ε-viniferin, quercetin, myricetin,

kaempferol and *trans*-cinnamic acid) in red wines, with no sample preparation. A preliminary application of the method in routine analyses of Southern Brazilian red wines is also presented here.

Experimental

Chemicals and standards

Methanol, acetonitrile and formic acid were HPLC grade, and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagent grade ethanol was obtained from Merck (Darmstadt, Germany). Ultra-pure water (Milli-Q, Millipore, USA) was used to prepare all the solutions. Analytical standards of *trans*-cinnamic acid, *trans*-resveratrol, *trans*-ε-viniferin, quercetin, kaempferol and myricetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Malvidin-3-*O*-glucoside and (+)-catechin were purchased from Extrasynthese (Genay, France). All standards were of at least 94% purity.

A stock solution was prepared containing 200 μg mL^{-1} of each analyte, whose concentrations were corrected according to respective purities, and was diluted with ethanol 70% (V/V in water). Fractions of the stock solution were kept protected from light and stored at 4 °C for a week. The working standard solutions were prepared daily.

Samples of red wines

Tests were conducted with samples of commercial red wines from the Campanha Gaúcha region, the grapes for which were harvested between January and February 2012. The following wine samples derived from six different varieties of grapes were used: Cabernet Franc, Cabernet Sauvignon, Malbec, Merlot, Tannat, and Tempranillo.

Before analysis, all samples were stored at 20 ± 2 °C. Samples were filtered through a 0.45 μm hydrophilic PVDF (polyvinylidene fluoride) membrane (Millipore, USA), and used without dilution.

Method development

The method conditions were optimized by testing different solvents (acetonitrile and methanol), column types (octylsilane–C8 and octadecilsylane–C18), column lengths (15 and 25 cm), column packing particle sizes (2.6 and 5.0 μm) and elution gradients. The peaks of the chromatograms were evaluated by R_s (resolution) and TF (asymmetry), following suitability recommendations (Bliesner 2006; FDA US Food and Drug Administration 1994; Kazakevich and Lobrutto 2007; Shabir 2003).

The settings for DAD detector were experimentally established. For this, the t_R (retention time) and λ_{max} (maximum ultraviolet absorption wavelength) were determined for each analyte. Full system suitability was also checked, considering: k (capacity factor), R_s, TF and N (number of theoretical plates).

HPLC–DAD conditions

Determinations of SaF and *trans*-cinnamic acid were carried out by HPLC–DAD. A Shimadzu system (Kyoto, Japan) equipped with a DAD detector (SPD-M10AVP), two dual piston solvent delivery pumps (LC-10AD), a controller module (UFLC CBM-20A) and CLASS VP software (version 6.12) were used. The column selected was a C8 (vertical) 150 × 4.6 mm, 5 μm particle size, carbon load of 9%, surface area of 450 m^2 g^{-1} and pore size 10 nm. It was protected with a guard column of C8 material (Phenomenex). Detection was conducted at 276, 306, 322, 365, 371 and 374 nm, according to the λ_{max} of each analyte. The injection volume was 20 μL and the flow rate was 1 mL min^{-1}. Mobile phase A consisted of formic acid and water (1:1000, V/V) and mobile phase B consisted of methanol, formic acid and water (900:1:100, V/V/V). The linear gradient was: from 30 to 45% of B in 7 min, 45% of B for 7 min, from 45 to 55% of B in 1 min, from 55 to 65% of B in 9 min, from 65 to 90% of B in 1 min, 90% of B for 2 min, from 90 to 30% of B in 0.5 min, 30% of B for 2.5 min. The total time of analysis was 30 min.

Method validation

The analytical method described in the "HPLC–DAD conditions" section was validated for its intended use. The performance characteristics defined in the validation guidelines from the Food and Drug Administration (FDA US Food and Drug Administration 2001) and the Brazilian Health Surveillance Agency (ANVISA 2003) were followed.

Selectivity was evaluated by assessing peak purity and R_s. Purity was determined using features of the DAD detector managed by the CLASS VP software, as follows: overlay of the spectra at different peak points; calculation of the purity index (from 250 to 600 nm); evaluation of the contour graph; and plots of three-dimensional (3D) graphs. Peaks of compounds were considered pure when the purity index was higher than 0.99.

For a more consistent evaluation of selectivity, an Ultima API Q-TOF instrument (Micromass, Manchester, UK) was used and it was controlled by Masslynx software, version 4.1 (Waters, Milford, United States). The analyses were conducted with ESI (electrospray ionization), in positive mode scanning from *m/z* 150 to 1000. The cone and desolvation nitrogen supply (99.0% purity) was set at flow rates of 30 and 300 L h^{-1}, respectively. The

instrument operation conditions were: capillary voltage of 2000 V, cone voltage of 100 V, source temperature of 80 °C, desolvation temperature of 150 °C. TOF was set at 9100 V, and detector voltage (MCP) was 1900 V, in V mode. The *m/z* data were registered by comparison with malvidin-3-*O*-glucoside (*m/z* 493.14) and (+)-catechin (*m/z* 291.09) standards, both of which are common wine constituents.

Linearity was performed by analyzing of three calibration curves on different days. Each solution was injected in triplicate. The stock solution was used to prepare calibration curve solutions at six concentration levels, being all of them diluted in ethanol 70% (V/V in water) (see "Chemical and standards" section). For cinnamic acid the concentrations were 0.1, 1.0, 2.5, 5.0, 7.5 and 10.0 μg mL^{-1}, whereas for the other analytes (*trans*-resveratrol, *trans*-ε-viniferin, quercetin, kaempferol and myricetin) the concentrations were 1.0, 5.0, 10.0, 20.0, 30.0 and 40.0 μg mL^{-1}. Linear regression equations were calculated by the least squares method. The linearity was evaluated by ANOVA.

Sensitivity was assessed by LOD (limit of detection) and LOQ (limit of quantification). They were determined by the noise level of the chromatograms obtained from linearity studies (ANVISA 2003; FDA US Food and Drug Administration 2001).

Precision was determined by an intra-day test (repeatability) and an inter-day test (intermediate precision). Repeatability was conducted by spiking a sample (Cabernet Sauvignon) with three concentration levels of SaF standards, prepared as six replicates on the same day. Concentrations were: low (L, 2.5 μg mL^{-1} for cinnamic acid and 10.0 μg mL^{-1} for the SaF), medium (M, 5.0 μg mL^{-1} for cinnamic acid and 20.0 μg mL^{-1} for the SaF) and high (H, 7.5 μg mL^{-1} for cinnamic acid and 30.0 μg mL^{-1} for the SaF). Intermediate precision was determined by spiking the same concentration levels, prepared in triplicate on three different days. The precision of the results was expressed by %RSD (relative standard deviation).

Accuracy was assessed by a recovery study processed in the same way as the repeatability work. Calculations of accuracy were based on comparison of the theoretical and the measured concentrations.

Robustness was corroborated by deliberate method modifications. Investigation of little differences in the elution gradient was conducted (Tests 1, 2 and 3). Test 1 was carried out as described in the "HPLC–DAD conditions" section from 0 to 14 min and from 26 to 30 min, with changes from 50 to 65% of B in 11 min and from 65 to 90% of B in 1 min. Test 2 was performed using a change from 30 to 40% of B in 7 min, 40% of B for 7 min, from 40 to 55% of B in 1 min and then the same as described in the "HPLC–DAD conditions" section from 15 to 30 min. Test 3 was run according to "HPLC–DAD conditions" section from 0 to 14 min and from 26 to 30 min, varying only from 50 to 55% B in 1 min, from 55 to 65% B in 9 min, from 65 to 90% B in 1 min and keeping 40% of B over 1 min.

Another test for robustness consisted of the replacement of the column by another C8 column (ACE, 150 × 4.6 mm, 5 μm, 10 nm pore size, surface area of 300 m^2 g^{-1}). This column had similar specifications to the one used in the original method, except for its surface area and brand.

Method application

The practicability of the validated method was checked with real red wine samples from the Campanha Gaúcha region, as described under the "Samples of red wines" section. All quantified analytes of these samples had their peaks compared with standards in terms of t_R, UV/VIS (ultraviolet/visible) spectra and purity.

Statistical analysis

Recovery, precision, robustness, linearity assessments, ANOVA, determination of calibration curve and r^2 were calculated using Excel 2013 software (Microsoft Corp., Redmond, United States), being confirmed through OriginPro 8 software (v8.0724) (OriginLab Corp., United States).

Results and discussion

Development of HPLC–DAD method

In this study, the focus was on developing a very selective method for the analysis of the largest possible number of markers of SaF biosynthetic pathways in red wines using an accessible technique (HPLC–DAD). To accomplish this goal, the method was exhaustively tested under the conditions presented in Table 1, and then optimized. Observations on the performance of the system suitability are also presented, namely R_s and TF for each methodological variation. R_s, which measures the separation between two peaks, is recommended to be over 2.0 (FDA US Food and Drug Administration 1994). The R_s values between most analytes and the adjacent peaks were at least 2.0, ensuring an adequate chromatographic separation (Table 2). The R_s of resveratrol was close to 2.0. However, some authors recommend acceptance criteria with R_s less than 1.5 (Bliesner 2006; FDA US Food and Drug Administration 1994). Considering the high purity of the resveratrol peak, the R_s close to 2.0 did not cause error of integration.

Table 1 HPLC conditions during SaF and *trans*-cinnamic acid method development

Column				Eluent[b]	System suitability[d]	
Phase	Dimensions (mm)[a]	Particle (µm)	Pore size (nm)	Organic[c]	TF	R_s
C18	250 × 4.6	5.0	12	ACN	>2.0	<1.5
C18	250 × 4.6	5.0	12	MeOH	≤2.0	<1.5
C18	150 × 4.6	5.0	12	ACN	>2.0	<1.5
C18	150 × 4.6	5.0	12	MeOH	≤2.0	<1.5
C18	150 × 4.6	2.6	10	ACN	>2.0	<1.5
C18	150 × 4.6	2.6	10	MeOH	>2.0	<1.5
C8	150 × 4.6	5.0	10	ACN	≤2.0	<1.5
C8	150 × 4.6	5.0	10	MeOH	≤2.0	>2.0

[a] Length and internal diameter of column

[b] Same gradient conditions as described in "HPLC–DAD conditions" section. Use of methanol (MeOH) or acetonitrile (ACN)

[c] Aqueous/organic ratio was constant. Organic phase was always 90% (V/V in water), and formic acid was added to this to give 0.1% V/V. Aqueous phase was fixed (formic acid 0.1% V/V)

[d] System suitability in terms of TF and R_s, with acceptable values of ≤2.0 and >2.0, respectively (FDA, US Food and Drug Administration 1994)

Table 2 Analytical characteristics and selectivity (values ± SD) of the HPLC–DAD method for SaF and *trans*-cinnamic acid standards, according to section "HPLC–DAD conditions"

Parameters	Recomendation[a]	*Trans*-cinnamic acid	*Trans*-resveratrol	*Trans*-ε-viniferin	Kaempferol	Quercetin	Myricetin
Detection characteristics[b]							
t_R (min)	t_R ± 1.0	23.38 ± 0.16	15.63 ± 0.20	21.78 ± 0.08	26.65 ± 0.21	22.53 ± 0.13	16.91 ± 0.32
λ_{max} (nm)	–	276	306	322	365	371	374
System suitability[c]							
k	>2.0	13.23 ± 0.05	8.57 ± 0.05	12.50 ± 0	15.47 ± 0.05	12.67 ± 0.30	9.37 ± 0.05
R_s	>2.0	2.25 ± 0.08	2.18 ± 0.04	4.38 ± 0.52	9.22 ± 0.09	7.13 ± 0.50	3.05 ± 1.42
TF	≤2.0	1.0 ± 0	0.97 ± 0.05	0.92 ± 0.04	1.05 ± 0.05	1.03 ± 0.05	1.02 ± 0.04
N	>2000	71202 ± 764	11872 ± 461	54081 ± 3098	41161 ± 1018	42283 ± 444	8923 ± 326
Selectivity[c]							
t_R (min)	t_R ± 1.0	23.06 ± 0.05	15.33 ± 0.08	21.60 ± 0.03	26.34 ± 0.03	22.22 ± 0.02	16.58 ± 0.08
λ_{max} (nm)	–	276	306	322	365	371	374
Purity	–	>0.99	1.00	1.00	>0.99	>0.99	>0.99

[a] According to Bliesner (2006), FDA US Food and Drug Administration (1994) and Shabir (2003)

[b] Determined by DAD detector for each analytical standard (Fig. 2a)

[c] Determined at λ_{max} of each SaF of a spiked wine (n = 6, low level = 10 µg mL^{-1})

The selectivity using a DAD detector has been previously reported, but with the use of an UPLC instrument (Silva et al. 2011). In addition, the TF of a peak should be equal to, or less than, 2.0 in order not to have tailing complicate the integration (Bliesner 2006; FDA US Food and Drug Administration 1994).

Guidelines require that the system suitability is shown to be acceptable before method validation (ANVISA 2003; FDA US Food and Drug Administration 2001). The only tested method that was in compliance with this requirement was using a C8 column and an organic mobile phase composed of methanol (see "HPLC–DAD conditions" section). Given its suitability, this method was chosen to be validated. Detection characteristics of this method, like t_R and λ_{max} are described in Table 2. Since the analytes studied all have a very strong chromophore, they also proved to have high enough molar absorptivity to be detectable by UV (Kazakevich and Lobrutto 2007). Nevertheless, the method is suitable for the DAD detector instead of a UV/VIS one, since the specific λ_{max} of each analyte is required for their detection. SaF and *trans*-cinnamic acid have different properties of λ_{max} and polarity,

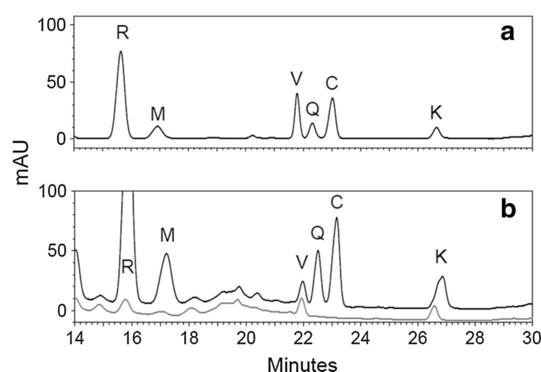

Fig. 1 HPLC–DAD chromatograms obtained according to parameters defined in "HPLC–DAD conditions" section: standard mixture with 10 μg mL^{-1} of each analyte at 306 nm (**a**); overlaid at 306 nm for unspiked red wine (*bottom line*) and red wine spiked (*top line*) with a standard mixture of analytes (**b**). Peak identifications: *trans*-resveratrol (R), myricetin (M), *trans*-ε-viniferin (V), quercetin (Q), *trans*-cinnamic acid (C) and kaempferol (K)

evidenced by different t_R values. In this study, such specific differences were fully explored to separate each analyte and to obtain a pure peak.

HPLC–DAD chromatograms at 306 nm demonstrated good peak separation (Fig. 1) allowing suitable identification of each peak (Fig. 2). The chromatogram of a solution containing a mixture of standards at 10 μg mL^{-1} is presented in Fig. 1a. A comparison of a typical red wine chromatogram with a spiked red wine (10 μg mL^{-1} of analytical standards) is illustrated in Fig. 1b, recorded at 306 nm. The analytical profile of each analyte was then established using the developed method.

The system suitability of parameters under the test conditions is also described in Table 2. The obtained values for all analytes in the low level spiked wine (2.5 μg mL^{-1} for cinnamic acid, and 10 μg mL^{-1} for other SaF) were according to recommendations (FDA US Food and Drug Administration 1994).

The use of a C8 column for SaF and *trans*-cinnamic acid analysis is different to most published HPLC methods, that use C18 phase columns instead (Careri et al. 2004; Cotea et al. 2012; López et al. 2001; Malovaná et al. 2001; Tenore et al. 2011). Determination of *trans*-resveratrol has been conducted with a column similar to the one used in this research (Zhou et al. 2004), i.e., it had the same technical specifications, but that study used an isocratic elution. The columns with a C8 phase are less hydrophobic than those with a C18 phase. Compared to C18, a C8 column decreases the retention of moderately polar compounds (Kazakevich and Lobrutto 2007), like SaF and *trans*-cinnamic acid. These characteristics were fully explored and found to give suitable separation and represented an advantage in method optimization. Then, a C8 phase column may be a good option to obtain the desirable

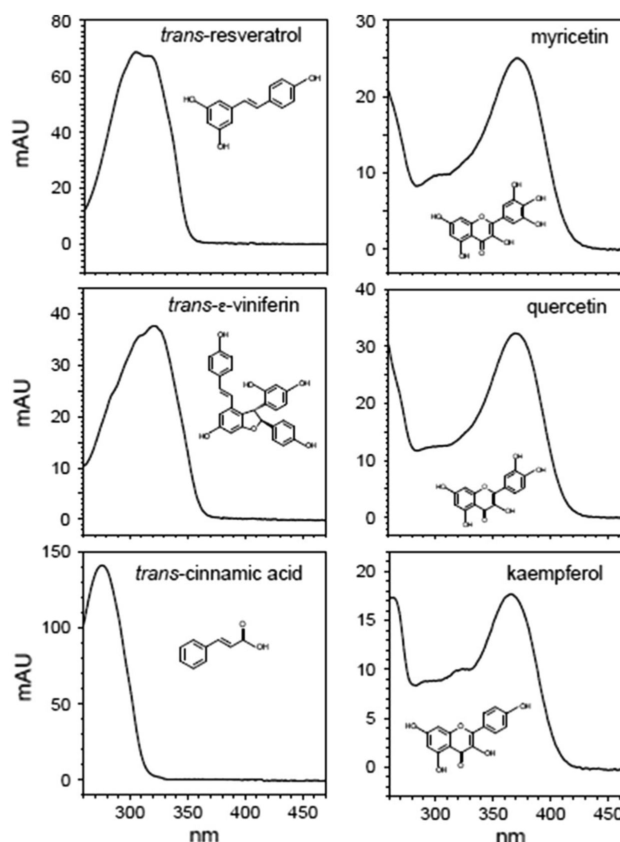

Fig. 2 Set of UV spectra for all the analytes, with their corresponding chemical structures

system suitability, selectivity and a fast chromatographic run for the HPLC standards (30 min).

Method validation

The method was considered suitable in terms of the performance characteristics defined by FDA US Food and Drug Administration (2001) and ANVISA (2003) guidelines, namely: selectivity, linearity, LOD, LOQ, precision, accuracy and robustness. Calibration results are reported in Table 3. Precision, accuracy and robustness are exposed in Table 4.

The primary method selectivity was verified by the classic crossing DAD data of UV spectra (Fig. 2), purity index (at least 0.99) and t_R (standard deviation-SD < 1) of the analytes. Previous methods have used only this simplified approach, or something even less rigorous, to check the selectivity (Castellari et al. 2002; Cotea et al. 2012; Kerem et al. 2004; Lima et al. 1999; Malovaná et al. 2001; Paulo et al. 2011; Pereira et al. 2010; Pérez-Magarino et al. 2008; Villiers et al. 2004). Some of these published chromatograms had critical regions of elution with wide unknown peaks and baseline deviation, as demonstrated in Fig. 1. This does not impair the use of the method, but

Table 3 Calibration values of the HPLC–DAD method (defined on section "HPLC–DAD conditions") for SaF and *trans*-cinnamic acid standards

Calibration[a]	Recommendation[a]	*Trans*-cinnamic acid	*Trans*-resveratrol	*Trans*-ε-viniferin	Kaempferol	Quercetin	Myricetin
Range (μg mL^{-1})	–	0.1–10.0	1.0–40.0	1.0–40.0	1.0–40.0	1.0–40.0	1.0–40.0
r^2	>0.99	0.9966	0.9972	0.9940	0.9954	0.9916	0.9952
Slope ± SD	–	254,231 ± 1633	167,034 ± 4180	66,919 ± 1752	123,799 ± 4113	87,227 ± 5055	89,030 ± 1366
Intercept ± SD	–	27,071 ± 1389	−35,370 ± 16851	−66,727 ± 4698	−182,808 ± 10076	−134,573 ± 7879	−119,958 ± 6143
Linear regression[b]	>9.33	9254.7	4739.2	3586.6	5055.0	2300.3	4599.3
Linearity deviation[b]	<5.41	4.96	0.27	2.32	2.82	1.87	2.50
LOD (μg mL^{-1})	–	0.02	0.33	0.23	0.27	0.30	0.23
LOQ (μg mL^{-1})	–	0.05	1.01	0.70	0.81	0.90	0.69

[a] Three calibration curves, in three different days. Calculations were performed accordingly to FDA, US Food and Drug Administration (2001) and ANVISA (2003)

[b] Calculated by least squares regression, with $p < 0.01$ ($n = 18$)

during validation it is recommended that a thorough knowledge of the interferences and their identities and t_R values be acquired (ANVISA 2003; FDA US Food and Drug Administration 2001). For these reasons, we conducted an exhaustive exploration of the DAD and QTOF data obtained from the chromatograms of red wines analyzed by the developed method. Chromatograms recorded at 525 nm (Fig. 3a), in the visible region, suggested the presence of anthocyanic pigments, according to a well know UV/VIS profile (Burns et al. 2002; OIV International Organization of Vine and Wine 2015). The three-dimensional graph (Fig. 3b) also illustrates an intense absorption (mAU) where these interferences elute through a range of t_R: from 0 to 15.0 min, from 19.0 to 21.3 min and after 28 min. QTOF data confirmed the interference of anthocyanic pigments and other phenols in these three t_R ranges, showing higher total ion counts through the chromatogram and also their characteristic *m/z* for molecular ions and fragments in positive mode (ESI^+) in accordance with previous publications (Burns et al. 2002; De Rosso et al. 2012; Flamini 2003; Gordillo et al. 2012; OIV International Organization of Vine and Wine 2015; Wu et al. 2005). The main interferences occurred from 0 to 15.0 min, where the combined mass spectra registered *m/z* 291.09, 579.13 and 867.18 which are compatible with wine flavanols (Fig. 3c). Trace levels of some nonacylated anthocyanins (*m/z* 479.08 and 465.09) were also detected through the first 15 min (Fig. 3d). From 19.0 to 21.3 min, a high quantity of malvidin-3-*O*-glucoside (*m/z* 493.14, and its fragment *m/z* 331.10) and peonidol-3-*O*-glucoside (*m/z* 462.14) were found (Fig. 3e). Additionally, the sensitivity of QTOF, over that of the DAD, enabled the detection of acetylated (*m/z* 505.14, 535.15) and coumarylated (*m/z* 639.17) anthocyanins that seem to appear after 28 min (Fig. 3f).

Anthocyanins, which are responsible for the color of red wines, and flavanols were the major interfering compounds in this analysis of SaF and *trans*-cinnamic acid in red wines. To ensure system suitability, it was necessary to identify a safety region to perform selective determinations of SaF and *trans*-cinnamic acid by direct injection of red wine in HPLC, that is: from 15.0 to 19.0 min and from 21.3 to 28 min. Absorptions by the interferents did not occur in the visible range at the t_R defined for analysis.

Linearity was observed for all analytes, over their concentration ranges and $r^2 > 0.99$. ANOVA confirmed a linear relationship ($F_{calc} > F_{tab}$, $p < 0.01$) and no deviation from linearity ($F_{calc} < F_{tab}$, $p > 0.01$). The calibration curves covered the range of analyte concentrations normally found in red wines (Adrian et al. 2000; Castellari et al. 2002; Fontana and Bottini 2014; Lima et al. 1999; López et al. 2001; Malovaná et al. 2001; Vitrac et al. 2005). Linearity, LOD and LOQ for each SaF and *trans*-

Table 4 Precision, accuracy and robustness of the HPLC–DAD method for SaF and *trans*-cinnamic acid analysis in cabernet sauvignon red wine

Parameters	Recommendation[a]	*Trans*-cinnamic acid	*Trans*-resveratrol	*Trans*-εε-viniferin	Kaempferol	Quercetin	Myricetin
Repeatability (%RSD)*							
L	<15	1.98	1.78	1.85	3.55	4.63	4.04
M	<15	0.94	1.90	1.35	5.11	4.44	4.15
H	<15	0.51	1.27	3.36	3.52	1.88	1.17
Intermediate precision (%RSD)*							
L	<15	5.56	5.65	3.20	8.03	7.48	5.24
M	<15	4.41	3.09	5.28	8.49	7.61	6.00
H	<15	3.78	4.58	4.97	9.16	8.48	5.37
Accuracy (%recovery)*							
L	85–115	94.66	101.30	106.27	101.32	97.94	101.52
M	85–115	102.57	102.48	100.98	103.62	98.70	99.77
H	85–115	102.07	105.86	106.95	87.40	90.12	86.70
Robustness (RT)							
Elution-test 1	–	23.91	17.03	22.48	27.54	22.97	17.80
Elution-test 2	–	23.15	17.76	21.72	25.74	22.98	19.02

The method is as described in the "HPLC-DAD conditions" section

* Spiked wine with three level of each analytical standard of SaF, defined on section "Method validation" (*L* low, *M* medium and *H* high)

cinnamic acid analysis are presented in the calibration curve data (Table 3).

Intra-day and inter-day precisions were acceptable because the %RSD between determinations did not exceed 15% (Table 4). In general, kaempferol and quercetin had higher %RSD than the other analytes. They are also the most hydrophobic of the compounds present in high concentrations, i.e., excluding cinnamic acid. Probably their solubilities in wine, with around 12% or 14% GL alcohol, were lower than those of the other analytes. However, such characteristic did not interfere in the method validation. As a result, the HPLC–DAD method was demonstrated to be precise for repeatability and intermediate precision.

Accuracy was demonstrated by suitable recoveries of all analytes (from 85 to 115%), as prescribed by the FDA US Food and Drug Administration (2001). The lowest determined values were for kaempferol, quercetin and myricetin, presumably for similar reasons explained above. The developed method was accurate for the required purpose, demonstrated no remarkable matrix effect (Table 4). Additionally, all identities were confirmed by UV spectra.

The developed method was robust, as some small modifications to its conditions did not affect the results (Table 4). Changing the gradient elution, the values of some t_R were modified. Nevertheless, purities were kept within acceptable values. Furthermore, quantifications did not deviate more than 5.0% (%RSD). Thus, the method was considered robust enough to be included in the routine repertoire of a laboratory.

Further and more significant alterations to the method were also tried by changing the C8 column for another one, but of a different brand and surface area. This second C8 column tested promoted separation of all compounds, except for viniferin and quercetin (Table 2). Viniferin had a t_R of 21.01 min and eluted very close to quercetin (21.18 min). UV/VIS detection of viniferin ($\lambda_{max} = 322$ nm) did not affect the detection of quercetin ($\lambda_{max} = 371$ nm). However, the opposite did not hold, since quercetin absorbs at 322 nm (Fig. 2). For this reason, viniferin could not be determined with this tested column without other alterations in the original method described under "HPLC–DAD conditions" section.

Application of HPLC method in red wine

In this study, the first goal was not necessarily to elucidate which of the wines from a region would be richest in their SaF markers. Before this comparison could be undertaken, it was necessary to put the current selective and validated HPLC–DAD method into practice to demonstrate its applicability as a feasible tool for the evaluation of red wines. Considering that wines are complex samples and their phenolic profiles are affected mainly by the grape variety, six varieties from the same region (Campanha Gaúcha) were tested to check the method selectivity. Therefore, the red wine samples were submitted to the method and their SaF markers were measured. Sample analyte peaks were comparable with those in standard profiles, including purity index (>0.99), t_R (SD<1) and UV/VIS spectra (Table 2; Figs. 1, 2).

The concentrations of five SaF and *trans*-cinnamic acid in red wines determined by this HPLC–DAD method are

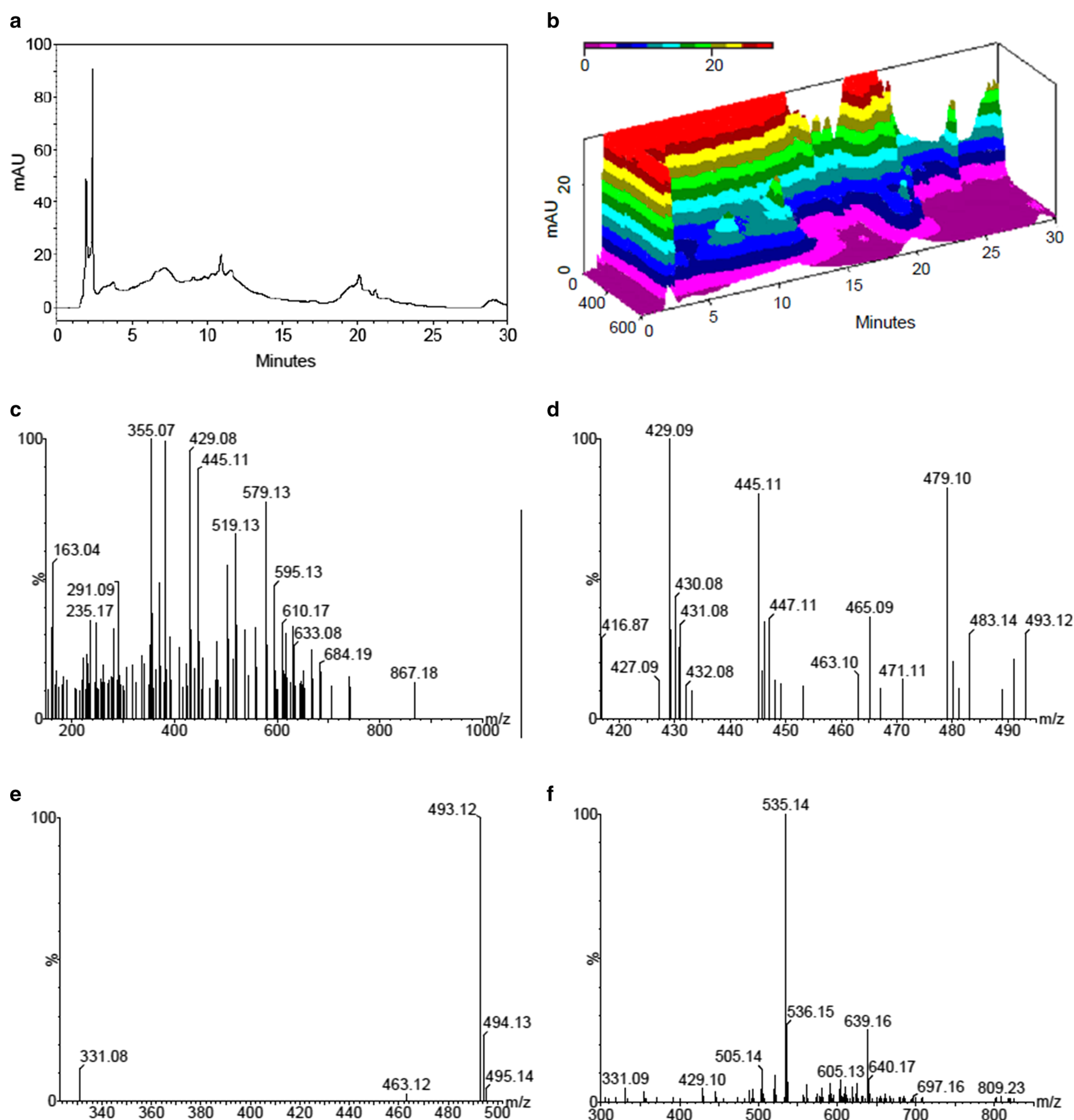

Fig. 3 Selectivity data for a sample of red wine showing no interference of anthocyanins with SaF and *trans*-cinnamic acid: HPLC–DAD chromatogram at 525 nm (**a**); 3D graph (**b**); mass spectrum of flavanol interferences, scanned from 0 to 15 min (**c**); mass spectrum of anthocyanic interferences, scanned from 0 to 15 min (**d**); mass spectrum of anthocyanic interferences, scanned from 19 to 21.3 min (**e**); mass spectrum of anthocyanic interferences, scanned from 28 to 30 min (**f**). For conditions of HPLC–DAD and QTOF, see "HPLC–DAD conditions" and "Method validation" sections, respectively

given in Table 5. The %RSD was higher for the lower SaF concentrations, but they could be reduced by employing an automated injector. The data presented were collected using a manual injector, taking into account analyst operation errors. Furthermore, *trans*-cinnamic acid and kaempferol showed the higher %RSD, but this is explicable by their low level and low solubility, respectively.

This is the first SaF characterization known for wines from the Campanha Gaúcha region. An exploration of the presented results, or the application of the same method for

Table 5 Concentration of SaF markers and *trans*-cinnamic acid in commercial red wines samples from Campanha Gaúcha, according to the method described under "HPLC-DAD conditions" section

Wine variety	Concentration[a] (μg mL^{-1})						Stilbenes[b]	Flavonols[c]	Markers of SaF[d]
	Trans-cinnamic acid	*Trans*-resveratrol	*Trans*-ε-viniferin	Kaempferol	Quercetin	Myricetin			
Cabernet franc	ND	ND	1.85 (3.06)	ND	4.01 (4.21)	2.37 (3.81)	1.85	6.38	8.23
Cabernet sauvignon	<0.05	3.65 (5.44)	3.32 (3.42)	2.01 (8.03)	5.19 (2.05)	7.86 (0.73)	6.97	15.06	22.08
Malbec	<0.05	2.37 (0.83)	1.95 (1.39)	1.78 (1.18)	4.64 (1.23)	8.07 (1.07)	4.32	14.49	18.86
Merlot	0.07 (8.13)	8.61 (4.36)	2.92 (0.41)	ND	<0.90	3.80 (4.56)	11.53	4.70	16.3
Tannat	<0.05	1.78 (2.76)	2.79 (4.34)	1.74 (4.70)	4.06 (0.94)	3.61 (4.23)	4.57	9.41	14.03
Tempranillo	<0.05	5.27 (3.62)	5.31 (2.42)	1.87 (4.45)	4.58 (5.46)	8.94 (3.96)	10.58	15.39	26.02

ND not detected

[a] Mean value and %RSD (in brackets), $n = 3$

[b] Sum of *trans*-resveratrol and *trans*-ε-viniferin

[c] Sum of kaempferol, quercetin, and myricetin

[d] Sum of all evaluated markers of SaF, where: ND = 0.00; <0.05 = 0.05; <0.90 = 0.90

new samples, may contribute for Certificate of Origin and strategies for the design of new products in this region (Ribéreau-Gayon et al. 2006). Here, the majority of the results were within the linear range of the method for each analyte.

Regarding each specific compound, viniferin, quercetin and myricetin were more frequently found than *trans*-resveratrol and kaempferol. Cinnamic acid was detected in a few samples and only quantified in one Merlot sample (0.07 μg mL^{-1}). The biosynthesis of SaF requires consumption of cinnamic acid, which is the first compound derived from phenylalanine (Pereira et al. 2010; Silva et al. 2011; Tenore et al. 2011). Generally, when this pathway to SaF is active, *trans*-cinnamic acid is present at low levels, whereas the SaF concentrations are high (Ribéreau-Gayon et al. 2006; Silva et al. 2011).

In general, the results presented in this study agreed with other studies previously reported for red wines (Adrian et al. 2000; Castellari et al. 2002; Fontana and Bottini 2014; Lima et al. 1999; López et al. 2001; Malovaná et al. 2001; Vitrac et al. 2005), which were similar in terms of the range of each SaF (μg mL^{-1}) present. More detailed comparisons among varieties or regions with those publications are meaningless, because the samples were analysed using different methods and conditions. Furthermore, other investigations proved that levels of SaF in wines are influenced by several factors like vineyard management, climatic conditions, vinification technology and grapevine variety (Carbonell-Bejerano et al. 2014). Despite this, clearly the developed method is usable and suitable for distinguishing samples through the analytes. This current HPLC–DAD method has several advantages over the previous published methods (Adrian et al. 2000; Aznar et al. 2011; Baptista et al. 2001; Careri et al. 2004; Castellari et al. 2002; Cotea et al. 2012; Fontana and Bottini 2014; Gómez-Alonso et al., 2007; Kerem et al. 2004; Lima et al. 1999; López et al. 2001; Malovaná et al. 2001; Pereira et al. 2010; Pérez-Magarino et al. 2008; Rastija et al. 2009; Revilla and Ryan 2000; Villiers et al. 2004; Vitrac et al. 2005). These include very simple sample preparation, high selectivity, fast analysis, full reported validation, with widely available instrumentation and it has been tested through analysis of different varieties of real wine samples. In summary, we have showed the compliance of this HPLC method to determine markers of SaF biosynthetic pathway in red wines.

Conclusions

QTOF provided the confirmation of analyte selectivity in a feasible HPLC–DAD method for the chemical characterization of the products of SaF biosynthetic pathways, through a simultaneous determination of *trans*-resveratrol, *trans*-ε-viniferin, quercetin, kaempferol, myricetin and *trans*-cinnamic acid in red wines. The developed method combines several methodological advantages to determine markers of bioactive SaF in red wines (Cabernet Franc, Cabernet Sauvignon, Malbec, Merlot, Tannat and Tempranillo). Validation results demonstrated that the proposed method is linear, sensitive, precise, accurate for each analyte, and robust. During validation, QTOF and DAD supported the establishment of the secure range of t_R to evaluate the analytes, avoiding overestimation of the results by coelution of the main interferents (anthocyanic pigments and flavanols). This method may be performed in any simple HPLC–DAD system, following the methodology recommendations in observing the analyte profiles on

DAD detectors. The sample preparation and handling are very simple and minimize analytical errors.

The method appears to be useful in routine analysis, being selective and feasible. The analysis of wines from the Campanha Gaúcha region, Brazil, proved that the method might be used for successful wine designs. Using those SaF as markers of the nutraceutical grade of a wine it is possible to study the quality of the product. It may also be employed to establish the optimal conditions of field management, vinification technologies and potential regions for each grape variety. Finally, the proposed technique can potentially contribute to agriculture and wine making, not only in the new Brazilian grape regions, because it may be extrapolated to other regions and samples. This HPLC–DAD method could be further adapted for the aglycone SaF and *trans*-cinnamic acid analysis of other wine varieties, grapes, grape by-products (e.g., juices), fruits, processed foods or beverages, following the specific sample preparation when necessary.

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