Journal of Chromatography B, 885–886 (2012) 43–49

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Optimisation of an HPLC method for the simultaneous quantification of the major sugars and organic acids in grapevine berries

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ARTICLE INFO

Article history:
Received 31 August 2011
Accepted 9 December 2011
Available online 20 December 2011

Keywords:
Sugars
Organic acids
Grapevine berries
High performance liquid chromatography

ABSTRACT

A high performance liquid chromatographic method was developed to profile major sugars and organic acids in grapevine berries. Sugars and organic acids in grapevine berries were extracted by chloroform/polyvinylpolypyrrolidone purification. The extracts were chromatographed on an Aminex HPX-87H ion-exchange HPLC column with 5 mM sulphuric acid as mobile phase. Chromatography was visualised via a diode array detector combined with a refractive index detector. The analysis was calibrated using external standard calibration and a novel equation was used to calculate the concentrations of malic acid and fructose from unresolved separation. For the method to be utilised for analysing a large numbers of berry samples, each sample was directly injected after sample extraction and the extraction step was downscaled to allow the use of small amounts of sample material. The concentrations of sugars and organic acids in grapevine berry samples were normalised to the internal standard concentrations obtained after extraction of an internal standard mixture. The analysis method exhibits a good precision and a high analyte recovery from samples spiked with the standard mixture and is suitable for the profiling of major sugars and organic acids in grapevine berry samples at different stages of berry development. This is the first report on the combined profiling of the major sugars and organic acids in grapevine berries using milligram amounts of plant material with direct injection after sample extraction.

1. Introduction

Sugars and organic acids are important primary metabolites that contribute to grapevine growth and berry development. These compounds are also considered key factors in grape and wine quality. The hexoses, glucose and fructose, as well as the organic acids malic and tartaric acid are the most abundant compounds contributing to the grape juice sweetness and acidity respectively [1]. Their concentrations and/or ratios vary during the berry development and maturation stages. Organic acids are produced in both the grape leaves and berries and start accumulating in the grapevine berry at early stages of berry development [1]. Hexoses are produced in leaves and are transported to the berry where a characteristic accumulation over time occurs in the vacuole of berry cells [1]. After véraison, the concentration of hexoses accumulate considerably in the berry while the acid concentrations start to decline with the exception of tartaric acid, which remains relatively constant throughout ripening [1,2]. The accurate quantification of these metabolites in grapevine berries at the different stages of berry development is important to follow berry development and ripening, specifically when evaluating the impact of viticulture practice on berry characteristics and subsequent wine properties.

Most methods for the analysis of sugars and organic acids in grapevine berries and wines that rely on high performance liquid chromatography (HPLC) have commonly used only the grape musts and/or juices with different sample extraction protocols [2–7]. Most of these extraction protocols are followed by clean-up steps prior to HPLC analysis to eliminate problems of co-elution of compounds of interest. These clean-up steps are often accomplished by fractionation through resin cartridges to separate the sugars and organic acids into two fractions before analysis [3,6,8]. Hunter et al. [8] developed a pre-analysis fractionation method that separated sugars and organic acid in two fractions before analysis from (1 g) whole, freeze-dried berries. The downside of fractionation methods is that it requires a significant amount of plant material and two HPLC runs per sample (increase time requirement). However, it has been shown that direct injection from grape juice and wine can provide an alternative for routine analysis with greater accuracy [9].

Here we describe an analytical HPLC method to profile the major organic acids and sugars by direct injection after sample extraction using small amounts (80–100 mg) of whole, frozen, deseeded and ground berries (pulp and skin). This method would be

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doi:10.1016/j.jchromb.2011.12.011

beneficial for researchers that are interested in accurately measuring the major sugars and organic acids from a single injection to follow berry development and ripening. The method is accurate with small amounts of plant material and thus particularly suited to applications where the sample availability is limited.

2. Materials and methods

2.1. Chemicals

Authentic standards of sugars and organic acids commonly found in grapevine berries (glucose, fructose, citric acid, tartaric acid, malic acid, succinic acid) as well as the compounds used as internal standards (ribitol and adipic acid), insoluble polyvinylpolypyrrolidone (PVPP) and acetonitrile (HPLC grade, UV cut-off 190 nm) were purchased from Sigma Aldrich Chemie (Steinheim, Germany). Sulphuric acid (H_2SO_4, 99.5% HPLC grade) was acquired from Merck (Midrand, RSA). The chloroform ($CHCl_3$, 99.0%) was provided by Merck Chemical (Wadeville, RSA). Analytical grade water was obtained from a MilliQ® water purification system.

2.2. Grapevine and wine samples

Grapevine berries from a Sauvignon Blanc experimental vineyard in the Elgin area of the Western Cape (South Africa) were collected at five stages of berry development: green (E-L stage 32), pre-véraison (E-L stage 34), véraison (E-L stage 35), post-véraison or ripening (E-L stage 36) and ripe/harvest (E-L stage 38). The E-L stages were determined as described in Coombe [10]. Pooled berry material was ground into a fine powder using an IKA-analytical mill (A 11 basic) and stored at −80 °C until extraction. Two wines (Cabernet Sauvignon and Sauvignon Blanc) were also tested after being diluted 10 times and filtered (0.22 μm nylon filter) before direct HPLC analysis (injection volume was 10 μL).

2.3. Standard preparation

A serial dilution of sugars (glucose and fructose) and organic acids (citric acid, malic acid, succinic acid and tartaric acid) was made by dissolving the required amount of authentic standard in deionised water (dH_2O). An equal volume (1:1, v/v) of the internal standard solution (ribitol and adipic acid) was added to each dilution series for standard preparation. All standards were filtered through a 0.22 μm nylon filter before HPLC analysis and the final internal standard concentration was 2 g/L. Each standard was analysed in duplicate for calibration and their concentrations ranged from 0.04 to 5 g/L for organic acids and from 0.1 to 20 g/L for sugars (see Table 1).

2.4. Extraction protocol

The extraction of sugars and organic acids from grapevine berry samples was adapted from a method described in Broeckling et al. [11] for *Medicago truncatula* cell cultures. The described method was modified for HPLC analysis and evaluated for its suitability for grapevine tissue to obtain a fast and robust method to reproducibly extract sugars and organic acids from frozen, homogenised grape berries using the minimum amount of tissue. For this, 1 g versus 80 mg frozen homogenised grape berry tissue were weighed off into 10 mL versus 800 μL of dH_2O containing 2 g/L of adipic acid and ribitol (as internal standards), respectively, and 5% (w/v) insoluble PVPP and vortexed for 5 min to homogenise. An equal volume of chloroform (10 mL versus 800 μL, respectively) was added to the mixture and the biphasic solvent was vortexed for 5 min to mix and incubated at 50 °C for 30 min with continuous shaking. After incubation, the microfuge tube was centrifuged at 17,500 × *g* for 10 min at room temperature to recover the upper aqueous phase containing the sugars and organic acids. The aqueous phase was re-centrifuged (as above) to remove any residual cell debris/particulate matter. The supernatant was transferred to HPLC vials and crimp-sealed for HPLC analysis. The sugar and organic acid concentrations obtained from 1 g of tissue and 10 mL extraction buffer was compared to the concentrations obtained from 80 mg of tissue and 800 μL of extraction buffer. Each grapevine sample was extracted in triplicate before HPLC analysis.

2.5. HPLC system, separation and quantification

An Agilent 1100 series HPLC system (Agilent Technologies©, Palo Alto, California, USA) equipped with a diode array detector (DAD) coupled to a refractive index detector (RID) was used to simultaneously separate and analyse organic acids and sugars. The system was run at 0.5 mL/min using an Aminex HPX-87H ion exchange column (300 mm × 7.78 mm) protected with a Bio-Rad guard cartridge (30 mm × 4.6 mm) composed of the same stationary phase and the column (not the guard cartridge) was thermostated at 55 °C. Mobile phases ranging from 2.5 to 22.5 mM H_2SO_4 in dH_2O with or without acetonitrile (6%) were tested to obtain the optimal chromatographic separation. ChemStation Rev. A.10.02 software (Agilent Technologies©) was used for data acquisition, peak integration and standard calibration. The injection volume was 10 μL. Organic acids were detected with the DAD at 210 nm while sugars were detected with the RID. An additional detection wavelength at 278 nm on the DAD was included for fructose. Peaks were quantified with external standard calibration based on areas and normalised with internal standard concentrations from the mock internal standard values. Adipic acid was used as internal standard for organic acids and ribitol for sugars. Three blanks (composed of only filtered dH_2O) were injected at the beginning of each run to condition the column. This was followed by two quality control (QC) standards (prepared independently from the external standards) both before and after every 10 samples for determination of the system variability, stability and precision. An additional two extracted internal standards (mock extractions) and two direct injection internal standard QCs were included in each sample run for normalisation purposes.

2.6. Derived equation to quantify the co-eluting malic acid and fructose

To determine the concentration of the co-eluting malic acid and fructose, different concentrations of both compounds were analysed separately with direct injection and their responses in both DAD and RID were compared to known concentrations.

The ratios of the fructose and malic acid responses, from the DAD and RID detection respectively, versus the known fructose and malic acid concentrations (k_1 and k_2) were calculated using Eqs. (1) and (2).

$$k_1 \frac{[\text{fru}]_{\text{DAD}}}{[\text{Fru}]} \quad (1)$$

$$k_2 \frac{[\text{mal}]_{\text{RID}}}{[\text{Mal}]} \quad (2)$$

where [Mal] and [Fru] are the known malic acid and fructose concentrations and $[\text{mal}]_{\text{RID}}$ and $[\text{fru}]_{\text{DAD}}$ are the RID and DAD responses for malic and fructose, respectively.

The ratios of the known fructose and malic acid concentrations versus the recorded fructose and malic acid concentrations (k_3 and

Table 1
HPLC retention time (Rt), detection system, response factor, calibration curve and instrument detection limit (IDL) of a standard solution of sugars and organic acids on an Aminex HPX-87H column using 5 mM H_2SO_4 as solvent.

No	Compounds	Rt (min)	Detection system	Response factor	Range (g/L)	Calibration curve				IDL (g/L)
						Y-intercept	Slope	R^2	SE	
1	Citric acid	9.48	DAD 210	0.59	0.04–5.00	8.37	1292.89	1.00	11.31	0.03
2	Tartaric acid	10.12	DAD 210	0.43	0.04–5.00	−1.43	1831.53	1.00	12.75	0.02
3	Glucose	10.77	RID	0.95	0.1–20.00	1917.69	130593.14	1.00	6960.81	0.16
4	Malic acid	11.35	DAD 210	0.65	0.04–5.00	−3.39	1204.04	1.00	8.43	0.02
5	Fructose DAD	11.45	DAD 278	30178.51	0.1–20.00	−0.23	4.13	0.99	0.42	0.30
6	Fructose RID	11.72	RID	0.81	0.1–20.00	386.80	152901.58	1.00	3334.52	0.07
7	Succinic acid	14.10	DAD 210	1.18	0.04–5.00	−6.13	684.57	1.00	4.48	0.02

k_4), from RID and DAD detection respectively, were calculated using Eqs. (3) and (4).

$$k_3 \frac{[\text{Fru}]}{[\text{fru}]_{\text{RID}}} \quad (3)$$

$$k_4 \frac{[\text{Mal}]}{[\text{mal}]_{\text{DAD}}} \quad (4)$$

where [Mal] and [Fru] are the known malic acid and fructose concentrations and $[\text{mal}]_{\text{DAD}}$ and $[\text{fru}]_{\text{RID}}$ are the recorded malic and fructose concentrations, from DAD and RID respectively.

From these ratios Eqs. (5) and (6) were obtained to assess the concentrations of fructose and malic acid in unknown samples:

$$[\text{Mal}]_{\text{exp}} = k_4([\text{mal}]_{\text{DAD}} - k_1 \times k_2([\text{fru}]_{\text{RID}} - k_2 \times [\text{mal}]_{\text{DAD}})) \quad (5)$$

$$[\text{Fru}]_{\text{exp}} = k_3([\text{fru}]_{\text{RID}} - k_2 \times k_4([\text{mal}]_{\text{DAD}} - k_1 \times [\text{fru}]_{\text{RID}})) \quad (6)$$

where $[\text{Mal}]_{\text{exp}}$ and $[\text{Fru}]_{\text{exp}}$ are the expected malic acid and fructose concentrations and $[\text{mal}]_{\text{DAD}}$ and $[\text{fru}]_{\text{RID}}$ are the recorded malic and fructose concentrations from DAD and RID respectively.

For this system the values for k_1, k_2, k_3 and k_4 were 0.05 ± 0.00, 0.67 ± 0.02, 1.22 ± 0.03 and 1.25 ± 0.02, respectively.

2.7. Method precision and recovery

The precision of the method was determined by calculating the coefficient of variation (%CV) of analyte concentration and retention time of six repeat extractions of véraison-stage grapevine berry samples analysed with the HPLC method. The calculation was done using the following Eq. (7):

$$\%\text{CV} = 100 \times \frac{\text{Stdev}}{\text{mean}} \times 100 \quad (7)$$

where Stdev is the relative standard deviation and the mean is the average values of the replicate analysis of each compound with HPLC.

The percentage recovery of the spiked standards was also evaluated in triplicate by adding known amounts of each compound into berry or wine samples in order to assess the effectiveness and accuracy of the method. The following Eq. (8) was used:

$$\%\text{Recovery} = \frac{[\text{analyte}]_{\text{spiked}} - [\text{analyte}]_{\text{unspiked}}}{[\text{analyte}]_{\text{added}}} \times 100 \quad (8)$$

where $[\text{analyte}]_{\text{spiked}}$ is the recorded amount of sugar/organic acid observed in the spiked sample, $[\text{analyte}]_{\text{unspiked}}$ is the amount of sugar/organic acid observed in the unspiked sample and $[\text{analyte}]_{\text{added}}$ is the amount of the sugar/organic acid standards added in the spiked sample.

3. Results

3.1. Method selectivity

Different aqueous H_2SO_4 solutions, ranging from 2.5 to 22.5, were initially tested as mobile phase to obtain the optimal conditions for the chromatographic separation. The 5 mM H_2SO_4 solution, recommended by Bio-Rad (column guidelines) was chosen and used for all experiments since it gave the best baseline stability and the lowest background noise (data not shown). Unfortunately, the co-elution between malic acid and fructose was not affected by varying the concentration of H_2SO_4. Attempts to use acetonitrile as an organic modifier in the mobile phase improved the resolution between malic acid and fructose however gave very high background noise and unacceptable baseline instability (data not shown).

3.2. Response linearity

Fig. 1 shows the chromatogram of the standard mixture of sugars and organic acids using an Aminex HPX-87H column operating with a 5 mM H_2SO_4 as mobile phase at 55 °C. The method allowed separation of all analytes except for malic acid and fructose, which co-eluted but could be detected by either RID and/or the DAD detectors. The internal standards (ribitol and adipic acid) eluted at 12.40 and 19.28 min, respectively, without interfering with the elution of the other standards. An additional absorbance peak at 278 nm was detected in the fructose authentic standard above 2 g/L and was therefore included for analysis (Fig. 1). The linear regression performed in each compound gave a regression coefficient (R^2) of >0.999 which shows a linear relationship between the chromatographic response areas and concentrations for all compounds (Table 1). The instrument detection limit (IDL) for each compound was measured based on the signal to noise ratio of 3 and ranged between 20 and 160 mg/L (Table 1).

3.3. Optimising sample extraction method

The extraction method adapted from Broeckling et al. [11] for *M. truncatula* cell cultures, was optimised to obtain the optimal conditions for HPLC analysis. One g versus 80 mg were evaluated to reproducibly extract sugars and organic acids from frozen, homogenised grape berries by comparing the ratios of the analyte amount (mg) per berry fresh weight (in g) after extraction. The 1 g of tissues gave similar results as the 80 mg indicating that the extraction method was as accurate as with >11 times less plant material and thus suitable for studies where a limited amount of plant material is available (results not shown).

3.4. Fructose and malic acid quantification

In order to quantify fructose and malic acid in unknown samples, the concentration values from DAD and RID signals, as well

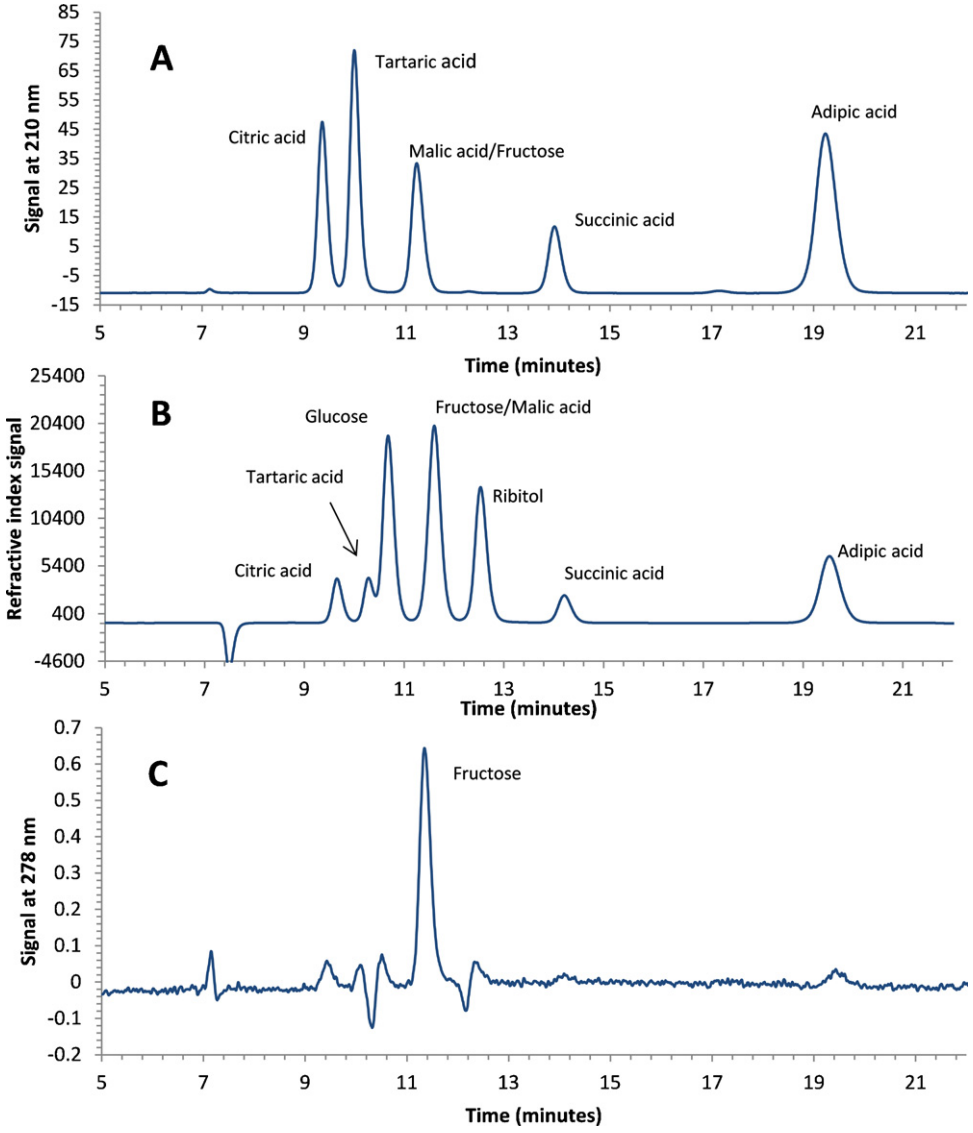

Fig. 1. Chromatogram of a standard mixture of sugars and organic acids detected using a DAD at 210 nm (A), a RID (B) and a DAD at 278 nm. Ten microliters of 0.625 μg/μL of each organic acid and 2.5 μg/μL of each sugar were injected through an Aminex HPX-87H (300 mm × 7.78 mm) column at 55 °C using 5 mM isocratic H_2SO_4 mobile phase. The different sugars and organic acids were identified by the elution time from standard addition.

as ratios based on known sample concentrations were used to set up equations as described in Section 2.6. To test these equations, different concentrations of malic acid and fructose mixtures were spiked to extracted grapevine berry and wine samples and analysed in duplicate with direct injection. The malic acid and fructose concentrations obtained using the equations were then compared to the known concentrations by measuring their percentage recovery in these matrices. The mean of percentage recoveries ranged between 94 and 106% for fructose and between 87 and 103% for malic acid in all these matrices (Table 2). This confirmed that the

Table 2
Percentage recoveries of malic acid (Mal) and fructose (Fru) after spiking to extracted berry or wine samples from corrected values using the equations versus known concentration values before analysis.

Samples	Stages/vintage		Mal–Fru mixtures (g/L)			
			0.00–1.25	0.63–0.00	0.63–2.50	Mean
Grapevine berries	Green	Mal	–	101.8 ± 6.0	100.1 ± 1.7	101.0
		Fru	97.2 ± 0.0	–	100.6 ± 0.7	98.9
	Véraison	Mal	–	97.7 ± 1.6	97.7 ± 0.1	97.7
		Fru	91.1 ± 0.1	–	96.2 ± 0.1	93.7
	Ripe	Mal	–	97.3 ± 0.4	102.6 ± 0.1	99.9
		Fru	98.0 ± 3.9	–	95.4 ± 1.8	96.7
Wines	Red wine (Cabernet Sauvignon)	Mal	–	90.0 ± 0.3	84.6 ± 0.3	87.3
		Fru	101.2 ± 0.4	–	96.7 ± 0.3	98.9
	White wine (Sauvignon Blanc)	Mal	–	102.9 ± 0.1	104.7 ± 0.1	103.3
		Fru	105.3 ± 0.5	–	108.8 ± 0.3	107.0

Table 3
System precision (%CV) and analyte recovery after standard addition on grape berry sample at different developmental stages.

No	Compounds	%CV	%Recovery				
			Developmental stages				
		Rt	Concentrations	Green	Véraison	Ripe	Mean
1	Citric acid	0.02	4.68	103.88 ± 0.14	100.58 ± 0.54	111.93 ± 1.15	105.46
2	Tartaric acid	0.02	0.95	87.66 ± 13.99	97.75 ± 3.75	104.63 ± 2.75	96.68
3	Malic acid	0.04	2.37	92.34 ± 5.78	93.43 ± 2.16	107.07 ± 2.12	97.61
4	Succinic acid	0.04	2.02	87.51 ± 7.28	100.62 ± 0.11	108.84 ± 1.38	98.99
5	Glucose	0.03	1.65	109.10 ± 0.13	103.82 ± 5.57	93.41 ± 7.06	102.11
6	Fructose	0.04	2.03	104.53 ± 4.36	103.23 ± 3.49	92.24 ± 8.23	100.00
7	Fructose 278	0.04	2.57	138.28 ± 2.13	120.65 ± 7.50	96.25 ± 10.61	118.39

equations are suitable to determine the fructose and malic acid concentration in grapevine berry samples. In addition to these equations, the values of fructose absorption at 278 nm were also used and compared with the calculated fructose at high concentrations (>2 g/L) in the subsequent experiments.

3.5. Method precision and analyte recovery

The precision of the method was verified by calculating the %CV of six repeat extractions of grapevine berry samples taken at the véraison stage after HPLC analysis to determine the variation in both the retention times and the concentration values of each component (Table 3). The %CV of the concentration and retention time values of major organic acids and sugars ranged between 1.6–4.7% and 0.02–0.04%, respectively, and were similar to values found in literature [7,12]. This indicated that the method was precise and highly reproducible for all compounds. The recovery of analytes after extraction from grapevine berries spiked with a mixture of standards was analysed in triplicate at green, véraison and ripening stages in order to assess the effectiveness and accuracy of the extraction step. The results confirmed that the separation and analysis conditions were accurate for all compounds (Table 3). The mean of the percentage recovery were 102.1% and 100.0% for fructose and glucose respectively, 105.5%, 96.7%, 97.6%, 99.0% for citric, tartaric, malic acid and succinic acid, in this order. For the recovery experiment, all grapevine berry samples were spiked with all compounds at the same time prior to extraction and HPLC analysis.

3.6. HPLC analysis of grapevine samples

The method was finally tested on grapevine berry samples at different developmental stages (green, pre-véraison, véraison, post-véraison/ripening and harvest [10]). Fig. 2 shows the chromatograms of sugars and organic acids extracted from grapevine berries at véraison. Major sugars and organic acids in grapevine berries were identified by their elution times as compared to authentic standard runs. The method allowed the simultaneous determination of all compounds of interest at different developmental stages of grapevine berries (Fig. 3). The quantification of malic acid and fructose concentrations in grapevine berries was achieved using the equation described in Section 2.6. The results show good correlation between the concentration of the calculated fructose (from RI) and fructose at 278 nm from véraison to harvest samples (ratios ≤ 1) (Fig. 3).

4. Discussion

The solvent system chosen for the analysis of sugars and organic acids in grapevine berries exhibited good baseline stability and low background noise although there was co-elution between malic acid and fructose. Problems of co-elution, particularly between malic acid and fructose is common in the analysis of sugars and organic acids of grape musts and wines as well as for many fruit juices and other fermented food products using HPLC [3,6–8,12–20]. This may be because fructose and malic acid are both small polar molecules with similar absorption and chromatographic properties. Castellari et al. [3] resolved this co-elution problem in the analysis of must and wine by using an aqueous H_2SO_4 solution in combination with organic modifiers such as acetonitrile. In this study, similar attempts to modify the eluting solvent by using acetonitrile as an organic modifier in the mobile phase improved the resolution between malic acid and fructose, but caused high background noise and poor baseline stability. The increased background noise reduced the sensitivity of the method for compounds present at low concentrations as is the case for sugars in the early stage of berry development (green stage) and malic acid in the harvest stage. Therefore, the current elution solvent was maintained and alternative solutions to quantitate malic acid and fructose were investigated.

Hunter et al. [8] proposed pre-analysis fractionation of the sugars and organic acid extracted from grape berry using an ion-exchange resin bed (Bio-Rex 5). The sugars (the neutral fraction) elute first in water while the organic acids (acidic fraction) are retained and elute with aqueous H_2SO_4. Although successful, this method necessitates two injections per sample and a large amount of grapevine berry material (1 g freeze-dried berries). In addition, the use of the resin cartridges does not allow the quantification of sugars in early stages of berry development (green and pre-véraison) where the sugar levels are very low (<10%, w/w) compared to the acids [8]. Since the purpose of this study was to develop a fast and easy method adapted to all stages of berry development suitable for large numbers of samples, using a small amount of sample material and where a single run per sample is desirable with minimum sample handling time, direct injection after sample extraction was selected and optimised.

Castellari et al. [3] suggested the combination of two detectors, DAD to monitor organic acids while sugars are detected with the RID. Dual detection during co-elution only works if one of the two compounds is exclusively detected in one detector and not the other. However, several studies (including this one) have shown that fructose also absorbs light, albeit less than malic acid, but ultimately leading to detection by both DAD and RID [3,18]. Lazaro et al. [17] proposed the use of both the DAD and RID simultaneously and a set of equations based on peak height values from both detectors to accurately quantify the co-eluting malic acid and fructose. In this study, concentration values from known samples from both detectors were used to set up equations to quantify fructose and malic acid in unknown samples. The results of investigating known malic acid–fructose mixtures using the equations showed a good percentage recovery for both compounds in different matrices (Table 2). This showed that the derived equations are suitable to calculate the

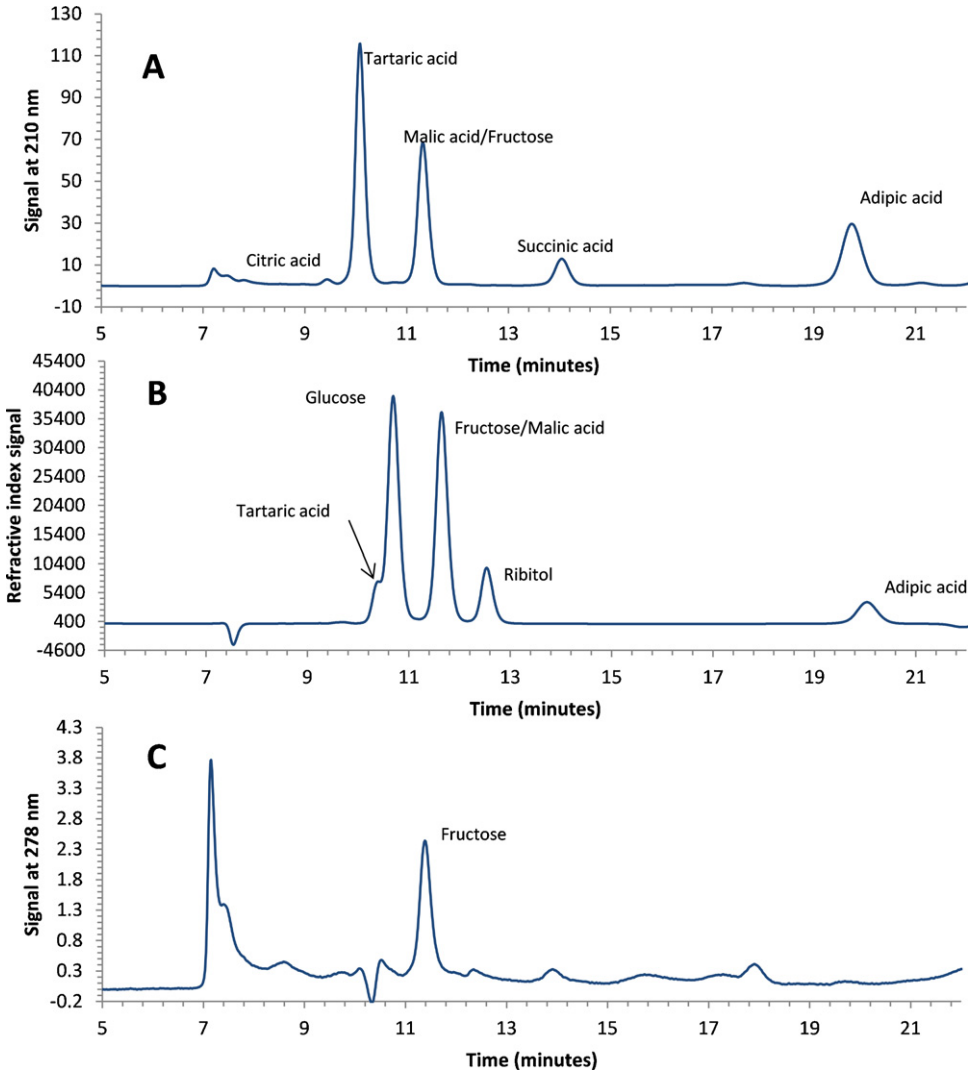

Fig. 2. Chromatogram of an extracted grapevine berry sample taken at véraison developmental stage detected using a DAD at 210 nm (A), a RID (B) and a DAD at 278 nm. The HPLC separation occurred on an Aminex HPX-87H column using 5 mM isocratic H_2SO_4 at 55 °C. The different sugars and organic acids were identified by the elution time from standard addition.

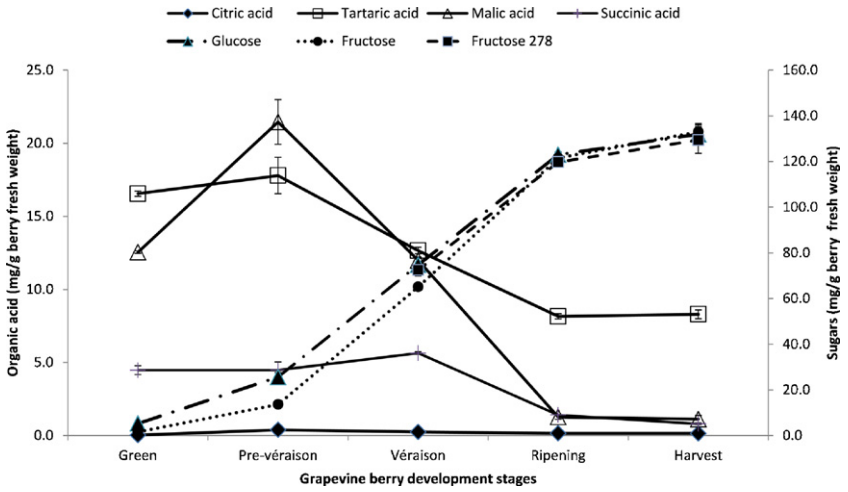

Fig. 3. Total sugar and organic acid content of extracted grapevine berries at the different developmental stages analysed with HPLC. Means represent an average of three extractions of the same sample and the bars represent the standard deviation values of the means.

concentrations of fructose and malic acid in unknown samples (Table 2).

Before analysing the grapevine berry samples, the extraction method was optimised to obtain the ideal conditions for HPLC analysis. To be suitable for the analysis, the extraction method was downscaled to allow the use of small sample size (>100 mg) with acceptable accuracy (>90%). The same analyte concentration per berry fresh weight was obtained after extraction from either 1 g or 80 mg of berry material (results not shown). This means that the extraction method is suitable to profile even a single berry (if required). According to Lund et al. [21] the development of berries in a bunch is independently regulated for each individual berry and is synchronised by other factors such as pigmentation but not the position on a bunch. An improved understanding of berry heterogeneity in a bunch necessitates a careful consideration of what a representative berry sample constitute and the possibility to analyse the components of single berries becomes attractive.

The determination of the %CV and analyte recovery is a valuable parameter to assess the precision of the system and accuracy of the analysis method. In this study, the values of the %CV and percentage recovery of berry extracts spiked with the mixture of standards at different developmental stages showed a good precision and high recovery (Table 3) and were similar to values in literature [3,13,15].

Analysis of grapevine berries at different developmental stages showed that the method allowed a simultaneous determination of all compounds of interest (Fig. 3). The developmental curve showed a decrease in organic acid concentrations and an increase in sugar concentrations as the berries progressed through ripening (Fig. 3) and were in accordance with previous studies [1,2,22,23]. The values of total organic acids as well as the ratios of glucose to fructose concentration were similar to values reported by Sabir et al. [2]. These results confirmed that the method was suitable for profiling major sugars and organic acids in grapevine berries during the different berry developmental stages.

5. Conclusion

This study presents an analytical HPLC method for the simultaneous analysis and quantification of the major sugars and organic acids present in grapevine berries. The extraction method was down-scaled for the use of small amounts of berry material, and a set of derived equations (based on concentrations from both the DAD and RID) is proposed to determine the concentration of malic acid and fructose from unresolved separation. The adaptation of this method allows the reliable analysis and quantification of major sugars and organic acids in grapevine tissues at different developmental stages.

Acknowledgements

This work was supported by grants from the South African Table Grape Industry (SATI), Wine Industry Network of Expertise and Technology (Winetech), the South African Technology, Human Resources for Industry Programme (THRIP) and South African National Research Foundation (NRF). Post-doctorial funding for EOA was provided by Carl Tryggers Stiftelse för Vetenskaplig Forskning, Sweden, and for LMG by NRF. Nina Lawrence (Institute for Wine Biotechnology) is thanked for technical support in the initial phase of the study and Dan Jacobson for experimental layout in the vineyard setting and participating in sampling from the vineyard. We wish to give special thanks to Antonio C. Ferreira (University of Porto, Portugal) for critical reading of the manuscript.

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