QUANTIFICATION OF GLUTATHIONE, CATECHIN AND CAFFEIC ACID IN GRAPE JUICE AND WINE BY A NOVEL ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD

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ABSTRACT

This research aimed at the development and validation of an ultra-performance liquid chromatography (UPLC) method for the quantification of glutathione (GSH) in grape juice and in white wine after derivatisation with *para*-benzoquinone. Catechin and caffeic acid, the most abundant phenols in white wine which have antioxidant effect, are also quantified in the same analysis. The first is the basic monomeric unit of grape and wine tannins and the second plays a relevant role in Grape Reaction Product (GRP) formation.

The analytical method proposed showed good linearity, repeatability and intermediate repeatability, as well as high recovery (> 85%). It was applied for the quantification of GSH, catechin and caffeic acid in South African juices (12) and white wines (43).

This novel method will have a large impact on the time and costs of the analyses for the wine industry through enabling rapid routine quantification of GSH, catechin and caffeic acid.

Keywords: caffeic acid, catechin, glutathione, grape juice, UPLC, white wine

Abbreviations: GSH: glutathione; pBQ: *para*-benzoquinone; MPA: 3-mercaptopropanoic acid.

1. Introduction

Glutathione (GSH) is an important antioxidant in white wine as it aids in decreasing aroma loss and the browning that occurs due to an oxidative processes in white wine. After pressing, the presence of polyphenol oxidase enzymes (PPO) can oxidize grape phenols into the respective *o*-quinones. During ageing, *o*-quinones are produced as a result of non enzymic oxidation (also known as chemical oxidation) of *o*-diphenols, of which some of the most susceptible *o*-diphenols include caffeic acid and catechin (Li, Guo & Wang, 2008). Both of the latter compounds are also the most abundant phenols in white wine (Margalit, 2004). The level of *o*-diphenols in wine is correlated to the browning of white wines, through enzymic oxidation to their respective polymerized *o*-quinones (Li et al., 2008; Margalit, 2004; Riberau-Gayon, Glories, Maujean & Dubourdieu, 2006). GSH plays a crucial role in the limitation of phenol oxidation during winemaking as it can react with caftaric acid, generating 2-S-glutathionyl caftaric acid, also known as Grape Reaction Product (GRP) (Salgues, Cheynier, Gunata & Wylde, 1986). In this way the formation of *o*-quinones and, consequently, the production of browning polymers, is limited.

Phenolic compounds such as caffeic acid and catechin exert an antioxidant effect on wine through their vicinal dihydroxy functions, which is able to form a stable semi-quinone radical that reacts with free radical oxygen reactive species that can be present under certain oxidative conditions. In this way, these compounds represent a natural wine preservative (Li et al., 2008; Waterhouse, 2002). Caffeic acid is one of the most common hydroxycinnamate acids in wine. Caftaric acid, the most abundant hydroxycinnamate found in grapes, consists of caffeic acid bound to tartaric acid. Caftaric acid is hydrolyzed naturally in wine of a few weeks old, liberating caffeic acid. The rate of this reaction is variable and is dependant on the pH of the wine and the presence of the hydroxycinnamate ester hydrolyze enzyme (Waterhouse, 2002). The content of caffeic acid in white wine can be as high as 70 mg L^{-1} (Castellari, Sartini, Fabiani, Arfelli & Amati, 2002; Landrault, Poucheret, Ravel, Gasc, Cros & Teissedre, 2001; Makhotkina & Kilmartin, 2010). Catechin is the

basic monomeric unit of grape and wine tannins (Riberau-Gayon et al., 2006) and concentrations vary from 5 mg L^{-1} to 100 mg L^{-1} in white wine (Margalit, 2004). Its content is highly correlated with the grape pressing technique employed (Waterhouse, 2002). Moreover, skin contact can increase catechin concentration in wine (Cheynier, Rigaud, Souquet, Barillère & Moutounet, 1989).

GSH exerts a protective effect on certain wine aromas (Lavigne & Dubordieu, 2004). It may lead to lower *o*-quinone-thiol associations, by competing for the *o*-quinones, thereby leading to higher amounts of thiol-related aromas in wine. GSH can also preserve aroma compounds, such as isoamyl acetate (3-methyl-1-butyl acetate), ethyl hexanoate, and linalool (3,7-dimethylocta-1,6-dien-3-ol) during bottle storage (Papadopoulou & Roussis, 2008), especially if caffeic acid is present in wine at certain levels (Roussis, Lambropoulos & Tzimas, 2007). Moreover, GSH can reduce the formation of sotolon (3-hydroxy-4,5-dimethyl-2(5H)furanone), a compound responsible for the atypical ageing character of white wine (Lavigne & Dubordieu, 2004).

In grapes, GSH concentration can exceed 100 mg kg^{-1} according to grape cultivar, environmental conditions and viticultural practices (Cheynier, Souquet & Moutounet, 1989). The GSH content in juice ranges from 10 - 100 mg L^{-1} (Cheynier et al., 1989) and factors such as exposure to oxygen, tyrosinase activity and the grape skin maceration during pre-fermentation can affect its concentration (du Toit, Lisjak, Stander, Prevoo, 2007; Maggu, Winz, Kilmartin, Trought & Nicolau, 2007). The concentration of GSH in wine is lower than in juice and grapes and it ranges from 3 to 20 mg L^{-1} (Cassol & Adams, 1995; du Toit et al., 2007). In instances where the concentration of GSH in white wine exceeds 6 – 10 mg L^{-1}, both colour and aroma were better preserved during ageing and storage (Lavigne & Dubordieu, 2004). *Saccharomyces cerevisiae* can also affect the GSH content in wine during alcoholic fermentation as well as during the ageing on the lees (Lavigne, Pons & Dudourdieu, 2007).

The analytical methods reported in literature to quantify GSH in juice and wine are enzymatic methods (Cassol & Adams, 1995), high-performance liquid chromatography (HPLC) with fluorescence detection (Janěs, Lisjak & Vanzo, 2010; Marchand, & de Revel, 2010; Park, Boulton

& Noble, 2000), HPLC with tandem mass spectroscopy (HPLC-MSMS) (du Toit et al., 2007), capillary electrophoresis (CE) (Lavigne et al., 2007), as well as atomic absorption spectrometry (Bramanti, Cavallaro, Onor, Zamboni & D'Ulivo, 2008). In general, wine phenols are commonly quantified using HPLC (Fabios, Lopez-Toledano, Mayen, Merida & Medina, 2000; Mayén, Mérida, & Median, 1995; Peng, Iland, Oberholster, Sefton & Waters, 2002).

Recently UPLC (Ultra Performance Liquid Chromatography) has been developed and applied to wine analysis (Gruz, Novák & Strnad, 2008; Schwarz, Rodríguez, Guillén & Barroso, 2009), although not yet fully exploited for wine compounds. Although conventional HPLC yields very good separation for phenolic compounds in wine analysis it is, compared to UPLC, a considerably slower chromatographic technique with the added disadvantage of generation of large volumes of chemical waste.

Acccording to the van Deemter equation (van Deemter, Zuiderweg & Klinkenberg, 1956) a decrease in HPLC column packing particle size is proportional to the efficiency of the chromatographic process. In fact, the particle size is one of the most influential parameters on separation efficiency (Naváková, Matysová, & Solich , 2006). According to the Van Deemter band broadening model that describes the relationship between the linear velocity and the height equivalent to a theoretical plate (HETP), the plate dependent term is directly dependent upon the column particle diameter. Higher efficiency can be achieved by using particles with smaller diameter (smaller than 2μm) as it would significantly reduce HETP. Subsequently, an increase in the flow rate of the mobile phase does not have such a large detrimental effect on separation efficiency as would be the case with column particles of larger diameter (5 - 10 μm). However, the consequence of using columns packed with smaller particles is the generation of high system back-pressure that cannot be tolerated by conventional HPLC systems. In HPLC the maximum back-pressure is typically 35 – 40 MPa, while in UPLC back-pressures of higher than 100 MPa can be handled (Nakaova et al., 2006). Therefore, when using UPLC it is possible to use stationary phases with particle sizes smaller than 2 μm, and high mobile phase linear velocities resulting in a

significant increase in the speed of analysis, sensitivity and resolution in comparison with convetional HPLC (Jerkovich, Mellors & Jorgenson, 2003; Nguyenet al., 2007).

The aim of the present study was to develop a fast, robust and selective UPLC method using a photo array detector (PAD) for the simultaneous determination of GSH, caffeic acid and catechin in grape juice and white wine. The analysis made use of a previously described derivatization procedure where GSH reacts with *p*-benzoquinone (pBQ), to introduce chromophores making detection by UV possible (Tirelli, Fracassetti & De Noni, 2010). The simultaneous analysis of these three compounds would be beneficial as many studies investigated the interaction between GSH, caffeic acid and/or catechin in model solutions or real wine and future work still needs to be done on these interactions (du Toit, Marais, Pretorius & du Toit, 2006) To the best of our knowledge this is the first UPLC-PAD application that allows for the determination of GHS.

2. Materials and methods

2.1. Chemicals

HPLC grade methanol, trifluoroacetic acid (TFA), GSH, pBQ, (+) catechin, cysteine, caffeic acid, citric acid, 3-mercaptopropanoic acid (MPA) and acetaldehyde were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC water was obtained from a Milli-Q filtration system (Millipore Filter Cor., Bedford, MA, USA).

A synthetic wine solution containing 12% ethanol and 2.5 g L^{-1} tartaric acid was prepared, and the pH adjusted to 3.5 with sodium hydroxide (Merck Chemicals). In order to evaluate possible interferences of ethanol and sugar content, a citrate buffer 50 mM at pH 3.5 and synthetic wine solution spiked with GSH (1.5 mg L^{-1}) and it contained 100 g L^{-1} glucose and 100 g L^{-1} fructose were prepared.

2.2. Samples

GSH, catechin and caffeic acid determination was carried out on 12 South African white grape juice samples consisting of 9 Sauvignon Blanc, 2 Semillon and 1 Chenin Blanc juices. Additionally, 43 South African white wines, of which 32 wines were tank samples and 11 commercial wines, produced from 5 different grape cultivars (Chardonnay, Chenin Blanc, Sauvignon Blanc, Petillant Blanc and Semillon) from the 2004 - 2010 vintages were analyzed.

2.3. Preparation of the samples

Sulfur dioxide (SO_2) was added to juices and tank samples at levels of 1 g L^{-1} and 0.04 g L^{-1} respectively, in order to prevent oxidation. The samples were centrifuged (4 mL) (Centrifuge 5415 D, Eppendorf, Hamburg, Germany) at 14000 rpm for 5 minutes.

Two mL of the supernatant was added to 100 µL of 14 mg L^{-1} acetaldehyde, left for 15 minutes at room temperature and then derivatized with pBQ. The derivatization was conducted as described by Tirelli et al. (2010): one hundred µL of 43.2 mg L^{-1} pBQ was added to the sample and 1 mL of 53 mg L^{-1} MPA was added after 1 minute of mixing, in order to react with the excess pBQ. The reaction mixture was mixed again and then microfiltered (0.22 µm, PVDF, Millipore).

2.4. Calibration curves

Calibration graphs were obtained by spiking known amounts of GSH (0.3 mg L^{-1} – 100 mg L^{-1}), catechin (0.5 mg L^{-1} – 80 mg L^{-1}) and caffeic acid (0.5 mg L^{-1} – 50 mg L^{-1}) to juice, synthetic wine solution and white wine.

2.5. Validation procedure

2.5.1. Precision parameters

The analytical method response was evaluated by spiking increased concentrations of GSH, caffeic acid and catechin in juice and white wine. The added concentrations of caffeic acid and catechin

were 4 mg L^{-1}, 10 mg L^{-1} and 22 mg L^{-1} both in juice and white wine. The addition of GSH was carried out at 1.2 mg L^{-1}, 3 mg L^{-1} and 6.7 mg L^{-1} in white wine. Juice was spiked with 6.7 mg L^{-1}, 21.2 mg L^{-1} and 45.5 mg L^{-1}, GSH as well. Samples were analyzed in triplicate.

2.5.2. Recovery

The recovery was calculated by comparing six replicate determinations of spiked and unspiked samples in all matrices for all considered compounds. For caffeic acid and catechin in juice and white wine three different concentrations were spiked, 4 mg L^{-1}, 10 mg L^{-1} and 22 mg L^{-1}. GSH recovery was evaluated at 1.2 mg L^{-1}, 3 mg L^{-1}, 6.7 mg L^{-1} for white wine and 6.7 mg L^{-1}, 21.2 mg L^{-1}, 45.5 mg L^{-1} for the juice.

2.5.3. Limit of detection and of quantification

The LOQ was determined at a signal to noise ratio of 10:1and the LOD at a signal to noise ratio of 3:1. Baseline noise was calculated considering peak-to-peak measurement for 3 minutes in two parts of the chromatogram.

2.6. Ultra Performance Liquid Chromatography

The UPLC separation was performed with a Waters Acquity UPLC (Milford, MA) equipped with a binary solvent pump, an auto sampler and a photo array detector (PAD eλ) (Milford, MA). The column was a BEH-C18 column (1.7 µm, 100 x 1.7 mm, Waters). The mobile phases consisted of water/trifluoroacetic acid (0.05% v/v) and methanol; the elution gradient increased from 10% to 35% of methanol in 8.5 minutes (Table 1). Detection was carried out at wavelengths of 303 nm, 280 nm and 320 nm for GSH, catechin and caffeic acid, respectively. The injection volume was 2 µL and the column was maintained at 25°C.

187 2.7. Quantification of compounds

188 The compounds were quantified chromatographically by the external standard method in juice,
189 synthetic wine solution and white wine. Data acquisition and processing were performed by
190 Empower 2 software (Waters, Milford, MA).

191

192 3. Results and discussion

193

194 3.1. UPLC method development

195 Studying the evolution of GSH in wine is of value to the wine producer, as it plays an important
196 role in the anti-oxidant capacity of both juice and wines. In the presence of GSH the oxygen
197 consumption of both caffeic acid and catechin has been shown to increase and the complex role that
198 these compounds and sulphur dioxide play in real wine needs to be assessed further (Cheynier &
199 Van Hulst, 1988; Cheynier & Ricardo da Silva, 1991; Danilewicz, Seccombe & Whelan, 2008).
200 Phenolics such as caffeic acid and catechin also affect certain sensory aspects of white wines, such
201 as their oxidation sensitivity, colour and aroma. The rapid analysis of these compounds would thus
202 be advantageous to both researchers and wine producers. UPLC instead of conventional HPLC
203 offers a sensitive, rapid, reliable, robust and solvent saving analytical tool (Naváková et al., 2006).
204 It has already been shown to be well suited for several applications within wine chemistry (Gruz et
205 al., 2008; Schwarz et al., 2009). In this study UPLC is shown to be very well suited for the analysis
206 of GSH, caffeic acid and catechin in juice and white wine. Derivatized GSH, as well as catechin and
207 caffeic acid were separated using the optimized analytical conditions described above. The elution
208 times were 4.2 min, 7.5 min and 9.2 min for derivatized GSH, catechin and caffeic acid,
209 respectively, with the derivatized MPA eluting at 8.2 min. The derivatization of GSH with pBQ is
210 fast, accurate and straightforward; no purification step, other than centrifugation, for either juice or
211 white wine samples was necessary prior to the derivatization reaction. The short analysis time and

212 the rapid sample preparation allowed the compounds to be quantified in less than 20 minutes. All of
213 the compounds investigated were represented by baseline separated peaks. (Fig. 1).
214 Ethanol and sugar content did not affect the derivatization yield; the derivatization of 1.5 mg L^{-1}
215 GSH was performed in 50 mM citrate buffer containing 100 g L^{-1} glucose and 100 g L^{-1} fructose at
216 pH 3.5, as well as in a synthetic wine solution containing 100 g L^{-1} glucose and 100 g L^{-1} fructose.
217 No significant differences were observed in the GSH concentration values detected in these
218 solutions (data not shown).
219 Sulphur dioxide, commonly used in winemaking, can react with pBQ. The addition of acetaldehyde
220 was therefore an essential step to bind the sulphur dioxide to achieve complete derivatization of
221 GSH to *S*-glutathionyl-*p*-hydroquinone (GSH-HQ) enabling UV detection. The excess of pBQ was
222 removed by addition of MPA in order to avoid oxidation of the hydroquinone derivates as well as
223 the phenols in the matrix. The product of this reaction, *S*-mercaptopropionyl-*p*-hydroquinone
224 (MPA-HQ) could also be detected in the chromatogram, confirming that the excess pBQ was
225 removed. The linear range of quantification was determined by spiking juice, synthetic wine and
226 white wine with the compounds considered at six concentration levels. All of the samples were
227 prepared and injected in duplicate for juice, synthetic wine solution and white wine at each
228 concentration level. The method showed a linear response for added concentration of GSH ranging
229 from 0 mg L^{-1} to 100 mg L^{-1}, catechin ranging from 0 mg L^{-1} to 80 mg L^{-1} and caffeic acid ranging
230 from 0 mg L^{-1} to 50 mg L^{-1}; these concentrations ranges are in accordance with those previously
231 found in juice and wine, in accordance with the concentrations indicated in literature (du Toit et al.,
232 2007; Janěs et al., 2010; Makhotkina & Kilmartin, 2010; Margalit, 2004). The calibration graphs for
233 juice, synthetic wine and white wine, respectively are shown in Fig. 2. The regression coefficients
234 reported in Fig. 2 indicate good linearity, with R^2 values of between 0.996 and 0.999. For the
235 quantification of GSH, catechin and caffeic acid, no significant differences were found in the
236 response between the synthetic wine solution and white wine. The calibration curves' slopes were
237 compared by the F Test (p = 95%). As the high value of the intercept shows, higher absolute values

were detected because of the native GSH amount in juice and catechin and caffeic acid content in white wine.

3.2. UPLC method validation

The repeatability and the intermediate repeatability were determined as described under Materials and Methods. Higher GSH levels were spiked in juice than wine due to higher GSH concentrations occurring in juice than in white wine (Cheynier et al., 1989; du Toit et al., 2007; Janěs et al., 2010). The values presented in Table 2 and 3 thus correspond to the sum of the native content and those spiked for each compound. As can be seen in Table 2 and Table 3 for both juice and white wine, good repeatability were obtained.

3.2.1. Limit of detection and quantification

The LOD in both juice and wine was 0.017 mg L^{-1}, 0.014 mg L^{-1}, and 0.0026 mg L^{-1} for GSH, catechin and caffeic acid, respectively The LOQ in both juice and wine was 0.057 mg L^{-1}, 0.048 mg L^{-1} and 0.0088 mg L^{-1} for GSH, catechin and caffeic acid respectively. For GSH, LOD and LOQ were lower than those previously reported in the literature when HPLC equipped with fluorescence detector was used. Values of 0.06 mg L^{-1} and 0.03 mg L^{-1} for LOD for juice and white wine and LOQ values of 0.2 mg L^{-1} and 0.1 mg L^{-1} for juice and wine were reported (Janěs et al., 2010; Marchand & de Revel, 2010). The LOD concentration for both catechin and caffeic acid in white wine were also lower than found previously found (0.11 mg L^{-1} and 0.02 mg L^{-1}, respectively) (Castellari et al., 2002).

3.2.2. Recovery

The recovery was performed and calculated by standard addition of the analytes of interest to juice and white wine as indicated under ”Materials and methods”.

For GSH, the recovery in juice was 100.3% compared to 88.4% in white wine. This is not surprising, as juice is know to have significant higher levels of GSH than wine and confirms previous findings (du Toit et al., 2007; Janěs et al., 2010). The recovery for catechin was 98.7% and 111.3% in juice and white wine respectively, while that of caffeic acid was 111.3% and 85.8% in juice and wine. These recoveries achieved during evaluation of the method was in accordance with those reported in white wine for both catechin and caffeic acid when quantified by HPLC (Castellari et al., 2002; Russo, Andreu-Navarro, Aguilar-Caballos, Fernández-Romero & Gómes-Hens, 2008).

3.3. Quantification in juice and white wine

In order to evaluate if the method is suitable for use as a routine analytical tool, 12 grape juice samples and 43 white wine samples. (Table 4). As seen in Table 4, GSH concentrations in juice ranged from 1.10 mg L^{-1} to 42.33 mg L^{-1}, which correlates well with values previous reported (Janěs et al., 2010; Maggu et al., 2007). The investigated phenols ranged from 0.73 – 8.67 mg L^{-1} and 0.50 – 3.71 mg L^{-1} for catechin and caffeic acid, respectively.

In wine, as can be seen in Table 4, the highest GSH concentration detected was 27.41 mg L^{-1}, which was in general higher in the white wines of the 2010 vintage. During wine ageing, GSH concentrations are known to decrease, leading to lower concentrations of this compound in older wines (Lavigne et al., 2007) which could explain the higher levels we observed in the 2010 wine in general. The amounts we detected were also in agreement with previous findings (Janěs et al., 2010; Woraratphoka, Intarapichet & Indrapichate, 2007). The average GSH levels of 2010 Sauvignon blanc tank samples (10 mg L^{-1}) was higher than those of the 2010 Chenin blanc tanks samples (5 mg L^{-1}). Sauvignon blanc juice is often treated more reductively in South Africa, with the addition of N_2, CO_2 and higher levels of SO_2 to the juice, leading to a elevated preservation of GSH levels in the juice and resulting wines (du Toit et al., 2007; Marais, 1998). The catechin concentration ranged

288 from 1.20 mg L^{-1} to 23.01 mg L^{-1} and caffeic acid between 0.16 mg L^{-1} and 3.69 mg L^{-1}, once again
289 correlating well with values reported in the literature (Landrault et al., 2001; Margalit, 2004).
290 The low concentrations of these *o*-diphenols observed in the white wines analyzed could have a
291 positive influence during ageing, as high levels of these compounds contribute to non-enzymatic
292 browning of white wine (Li et al., 2008).
293
294 4. Conclusion
295 This novel UPLC method allows for the detection and quantification of GSH, catechin and caffeic
296 in one rapid chromatographic analysis. The GSH derivatization is fast and the preparation of
297 samples is fast, easy and no purification steps are required. The validation has shown good
298 repeatability, intermediate repeatability and linearity both in juice and in white wine, as well as a
299 good recovery in both of the matrices for all the compounds investigated.
300 The chromatographic method is also easy, sensitive, robust and fast, making it excellently suited for
301 routine analysis. The reduced analytical run time allowed for less solvent use, which reduces waste
302 generation, rendering the method more environmentally friendly and more cost efficient.
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Fig. 1: Chromatographic separation and detection at 280 nm of the three compounds in a model wine solution (A), must (B) and white wine (C). Peaks: 1: GSH-HQ, 2: catechin, 3: MPA-HQ, 4: caffeic acid.

Fig. 2: Calibration graphs in model wine solution (●), white juice (♦) and white wine (▲). A: GSH, B: catechin, C: caffeic acid.

Table 1: Mobile phases and elution conditions; A: water/trifluoroacetic acid 0.05% (v/v), B: methanol.

Table 2: Standard deviation of repeatability and intermediate repeatability in juice; SD: standard deviation, expressed in mg L^{-1}; RSD: residual standard deviation, expressed in percentage.

Table 3: Standard deviation of repeatability and intermediate repeatability in white wine; SD: standard deviation, expressed in mg L^{-1}; RSD: residual standard deviation, expressed as a percentage.

Table 4: Description of juices and white wines analyzed and its quantification of GSH, catechin and caffeic.