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Automated HPLC analysis of glutathione and thiol-containing compounds in grape juice and wine using pre-column derivatization with fluorescence detection

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Abstract

An easy and sensitive method for the analysis of glutathione (GSH) and other thiol-containing compounds in grape juice and wine has been developed and optimized. Following a pre-column derivatization of thiols with *o*-phthalaldehyde (OPA) and 2 aminoethanol, isoindole derivatives are separated on reversed-phase HPLC column and quantified by a fluorescence detector. The minimum detection limits for thiols are: GSH, 3.3 nmol/l (1 μg/l); cysteine, 22 μmol/l (2.7 mg/L); methanethiol, 0.27 μmol/l (12.8 μg/l); ethanethiol, 0.65 μmol/l (11 μg/l). The method yields linear responses up to 40 and 21 mg/l for GSH and cysteine, respectively. GSH levels in two varietal grape juices during fermentation varied from 0 (starting juice) to 2.1 mg/l (wine) in Sauvignon blanc, while the GSH in a Palomino sample with 1.28 mg/l in the juice increased to 5.1 mg/l in the wine. This automated pre-column derivatization of thiols followed by an automatic injection procedure is sensitive, reproducible and rapid, with a run time of 35 min.

Keywords: Grape juice; HPLC; Glutathione; Thiols

1. Introduction

Low molecular weight and hydrophilic compounds with one or more thiol functional groups, especially GSH (L-γ-glutamyl-L-cysteinylglycine), have received much attention owing to their physiological importance in living cells (Jocelyn, 1972; Larsson, Orrenius, Holmgen & Hannervik, 1983; Pheifer & Briggs, 1995; Vina, 1990). In analysis of 21 varieties of grapes, GSH ranged from 17.3 to 114.4 mg/kg (Cheynier, Souquet & Moutounet, 1989). In yeast, GSH accounts for about 1% of dry weight of *Saccharomyces cerevisiae* and represents more than 95% of the low-molecular-mass thiol pool (Elskens, Jaspers, Penninckx, 1991). GSH is essential for the proliferation of yeast cells, therefore, yeast mutants with deficiencies in GSH synthesis show extremely long generation times (Murata & Kimura, 1986). GSH serves as a storage compound which can be mobilized during starvation and/or reproduction, and is used in the synthesis of cysteine (Elskens et al.; Meister & Anderson, 1983). Despite the potential importance of GSH in yeast metabolism, it has not been studied in wines or fermenting musts. Similarly, although the volatile thiols, such as methanethiol or ethanethiol, contribute to off-odors in wines, very few studies have analyzed them because of the difficulty of analysis of trace levels of thiols.

In the analysis of thiols by HPLC, a pre-column derivatization using *N*-(9-acidinyl) maleimide resulted in the formation of multiple by-products due to the hydrolysis of fluorescent products (Takahashi, Nars, Heguro & Tuzimur, 1979). To circumvent this, Nakamura and Tamura (1981) used a post-column derivatization based on the reaction of thiols with *o*-phthalaldehyde (OPA) and primary amines. However, this technique required an additional dedicated post-column reaction system and its sensitivity was ultimately limited by the fluorescent background of the reagent. Recently, Sano and

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Nakamura (1998) reported a pre-column derivatization of thiols in human blood using HPLC chemiluminescence detection. This method also required an additional post-column derivatization of isoindole derivatives following separation by reversed-phase HPLC. A pre-column derivatization method using OPA as a derivatizing agent for fluorometric determination of thiols gave better sensitivity and reproducibility (Jocelyn & Kamminga, 1970; Mopper & Delmas, 1984) than methods using UV detection (Alegria Toran, Farre, Lagarda & Lopez, 1966; Yoshida, 1996). Although HPLC analysis of isoindoles formed by derivatization using OPA eliminated by-product formation, the isoindoles were unstable in the aqueous reaction mixture resulting in variable reproducibility (Mopper & Delmas, 1984). In this report a procedure for automation of the derivatization and analysis procedure for thiols was developed which permits immediate analysis of the isoindole derivatives. The automated protocol developed from the pre-column derivatization method of Mopper & Delmas (1984) is suitable for analysis of thiols in grape juice and wine, including GSH, L-cysteine, methanethiol (MeSH), and ethanethiol (EtSH).

2. Materials and methods

2.1. Reagents and chemicals

Deionized water (Millipore, Milford, MA) and HPLC-grade reagents and solvents were used for the mobile phases, reagents, and sample preparation. The mobile phases consisted of 50 mM sodium acetate, pH 5.7 (buffer "A") and methanol (buffer "B"). The two derivatizing reagents were 2 mg of *o*-phthalaldehyde (Aldrich Chemical, Milwaukee, WI) dissolved in 1 ml methanol and 2 μl of 2-aminoethanol (Aldrich Chemical) dissolved in 1 ml of 0.8 M sodium borate (pH 7.4). Standard thiols were purchased from following sources: GSH, and L-cysteine (Sigma Chemical, St Louis, MO); methanethiol and ethanethiol (Kodak Chemicals, Rochester, NY); hydrogen sulfide (Liquid Carbonic, Los Angeles, CA). *N*-acetyl-L-cysteine (Sigma Chemical) was used as an internal standard. The stock solutions of standard thiols (GSH: 3.25 mM (1000 ppm); cysteine 1.73 mM (210 ppm); 1 mM methanethol; 1 mM ethanethiol) were prepared in 5 mM sodium acetate buffer (pH 4) containing 0. 1 mM EDTA. The stock solutions were made fresh each week, and serially diluted with sodium acetate buffer to desired concentrations, immediately prior to derivatization. All reagents and samples were put into sample vials (1.5 ml) which had been previously purged with nitrogen gas shortly before sampling. The headspace was also purged with nitrogen gas immediately before sealing the vial with a Teflon-faced septum.

2.2. Grape juice and wine

Sauvignon blanc and Palomino grapes, grown in the University experimental vineyard in Davis, were used for this study. The crushed juices were fermented in 650 ml, batches at 23°C in small (1 l) laboratory fermentors which were stirred at 200 rpm (Applikon, The Netherlands). For analysis, 0.5 ml juice samples were taken from the juice prior to fermentation, daily during fermentation and from the finished wine. The 0.5 ml samples were diluted with 0.5 ml of 5 mM sodium acetate buffer (pH 4) containing 0.1 mM EDTA.

2.3. Derivatization procedure

The pre-column derivatization developed in this study is a modification of a manual analysis of thiols by reaction with *o*-phthalaldehyde and 2-aminoethanol described by Mopper & Delmas (1984) and of an automated amino acid analysis procedure (Schuster, 1988). Using the Hewlett-Packard autosampler which includes an on-line derivatization system, 2 μl of OPA were withdrawn from vial No. 1, the needle was washed with H_2O and 5 μl of grape juice or wine were withdrawn from the sample vial and the needle was washed again by H_2O. Finally, 2 μl of 2-aminoethanol were withdrawn and mixed for exactly 1 min by moving the reagents and sample volumes back and forth (two cycle) inside the autosampler's syringe capillary. The derivatized sample (total 9 μl) was then injected immediately by automatic injector for analysis. For each sample, this automatic derivatization procedure is performed just before injection.

2.4. Liquid chromatography

A Hewlett–Packard HPLC system (Model 1090) equipped with an autosampler which permits on-line derivatization was used for analyses. This automatic HPLC system was controlled by HP Chemstation (HP

Table 1.
Solvent gradient conditions. Buffer A = 0.05 M sodium acetate (pH5.7); Buffer B = 100% methanol

Time (min)	Buffer A (%)	Buffer B (%)
1	90	10
2	85	15
6	72	28
7	68	32
9	64	36
10	56	44
12	52	48
19	50	50
21	40	60
25	32	68
26	0	100
31	90	10

79994A). Using the gradient program for the mobile phases shown in Table 1, derivatives were separated on an Ultramex 3 C_{18} column (100 mm×4.6 mm I.D., 3 mm packing) (Phenomenex, Torrence, CA) and detected by a programmable fluorescence detector (Hewlett Packard, model 1046A) where wavelengths for excitation and emission were 340 and 450 nm, respectively.

2.5. Reproducibility and identification

Identification of unknown peaks was made by comparison with the retention time of known thiols. Standard curves were developed for quantifying the individual thiols. Triplicate analyses were performed using standards to determine both response linearity and reproducibility of the protocol. Methionine, which contains no thiol derivatives, was also examined to test for interference.

3. Results and discussion

For amino acid derivatization, 2-mercaptoethanol is generally used as a nucleophile. In thiol analysis, however, the test thiol compounds react as nucleophiles yielding the highly fluorescent isoindole derivatives by reaction with *o*-phthalaldehyde and 2-aminoethanol in aqueous solution and at mild basic pH. In thiol analysis, the sequence of the addition of reagents affects the yield of the reaction. As observed previously (Roth, 1971; Simon & Johnson, 1978), maximum sensitivity of this method was obtained by adding the experimental sample (containing thiols) to OPA, followed by the addition of the 2-aminoethanol. In this study, the reaction of thiols with 2-aminoethanol followed by OPA gave about 40% lower peak area.

The retention times (min) for authentic thiols were: GSH, 6.95; cysteine (Cys), 7.95; *N*-acetyl-L-cysteine, 8.98; MeSH, 18.46; EtSH 23.26; H_2S, 25.00 under the conditions used. Methionine, which does not have a thiol group, did not yield any fluorescent peak in reaction with OPA indicating no interference by the derivatizing chemicals and compound containing sulfur in functional groups other than thiols. Even at low concentrations, there was no interference from reagents or reagent by-products, in contrast to an earlier pre-column derivatization technique for thiols (Lindroth & Mopper, 1979),

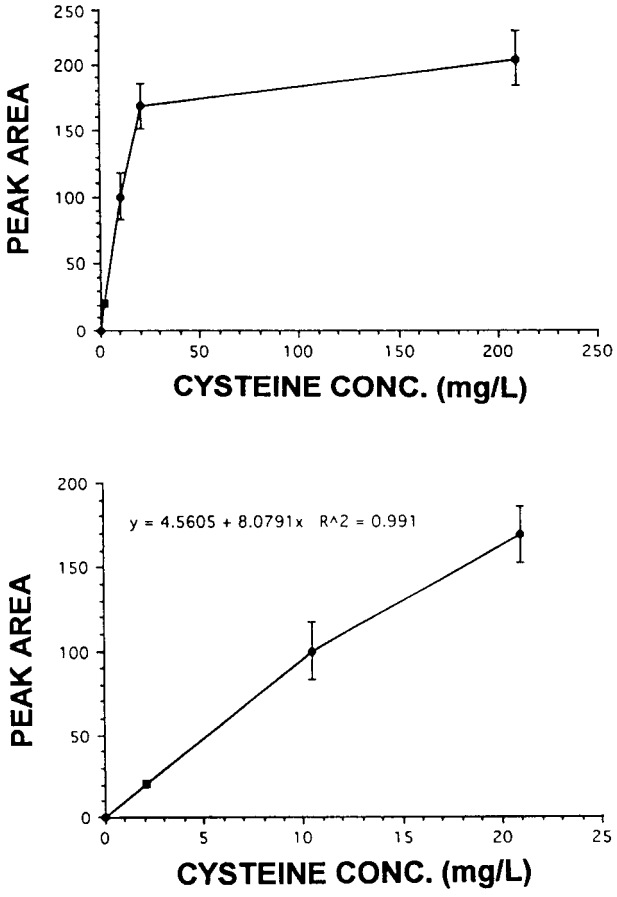

Fig. 1. Linearity of response for cysteine. Top: 0–200 mg/l; bottom: 0–21 mg/l. Barbell denotes standard deviation from triplicate analysis.

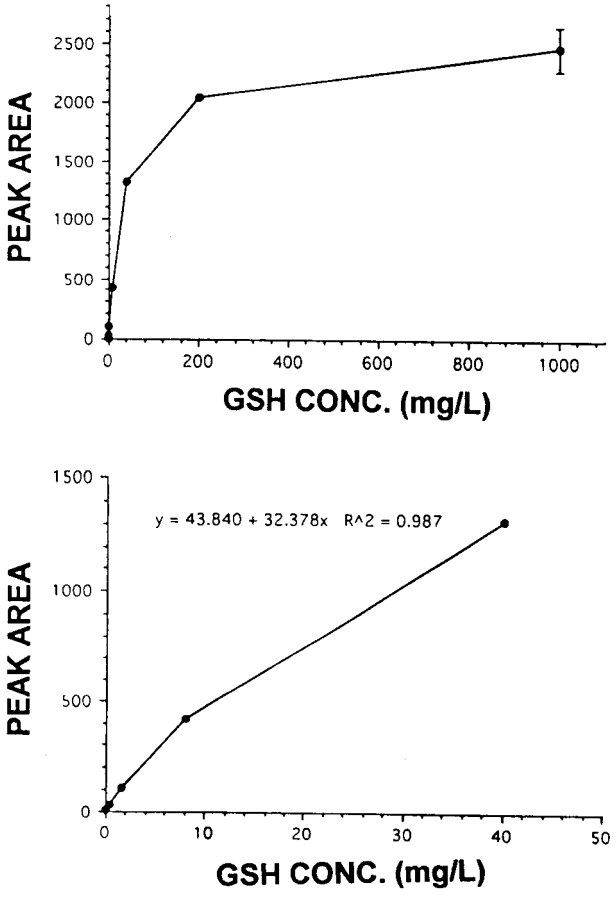

Fig. 2. Linearity of response for glutathione. Top: 0–1000 mg/l; bottom: 0–40 mg/l. Barbell denotes standard deviation from triplicate analysis.

Reproducibility of the analysis was calculated from triplicate analyses. The coefficient of variation was 0.83% at 40 mg/l for GSH, and 9.9% for cysteine at 21 mg/l level. Although the detection limits for MeSH, and EtSH were very low, their results were highly variable because of their high volatility. These thiols and other volatile sulfur compounds were reproducibly detected in these fermenting musts and finished wines by a gas chromatographic headspace analysis method reported elsewhere (Park, Boulton & Noble, 1999). Cysteine was shown to have a linear response up to concentration of 21 mg/l with a r^2 of 0.991 (Fig. 1). For glutathione, the response is linear up about 40 mg/l GSH ($r^2 = 0.987$), however, the response for GSH decreases above 40 mg/l (Fig. 2). This decrease in GSH response not the result of swamping of the detector, but may be due to non-thiol side-reactions with GSH (Nakamura & Tamura, 1981; Simon & Johnson, 1978). Reaction by-products were detected in analysis of samples containing GSH at concentrations above 40 mg/l as shown in Fig. 3. Using cysteine as a reactant, the isoindole derivative of cysteine was identified as one by-product. Six other peaks observed from analysis of higher concentrations of GSH were not identified. These byproducts did not form in analysis of other thiols even at high concentrations, possibly since other thiols had no reactive functional groups or had small side chains.

The maximum sensitivities (with a signal-to-noise ratio of about 2) were GSH, 3.3 nmol/l (1 μg/l); cysteine, 22 μmol/l (2.7 mg/l); methanethiol, 0.27 μmol/l (12.8 μg/l); ethanethiol, 0.65 μmol/l (11 μg/l). Mopper & Delmas (1984) reported a detection limit of 0.25 nM (with 100 μl injections) which was averaged over six thiols, hence their sensitivity, although similar, cannot be directly to compared the data in this study.

3.1. Analysis of grape juice and wine

Fig. 4 shows the typical traces from analysis of Palomino grape must and wine. Peak No. 1 is GSH, No. 2 is tentatively identified as γ-glutamylcysteine, an enzy-

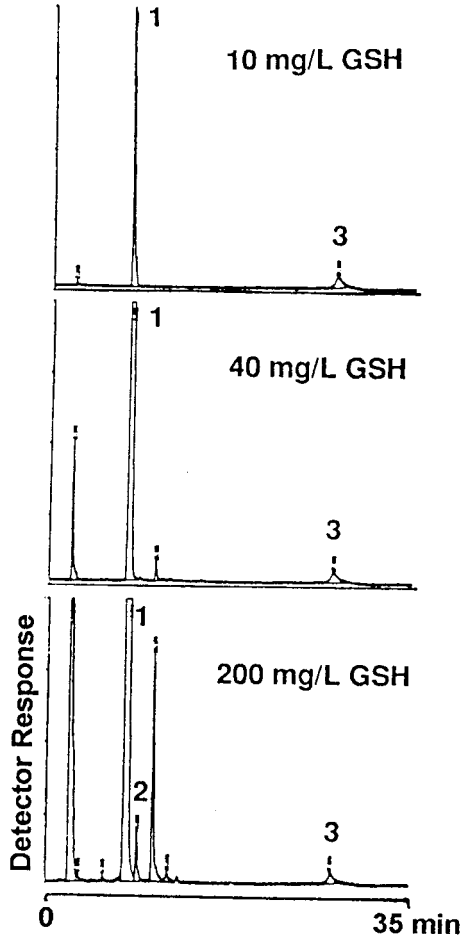

Fig. 3. Formation of reaction products by GSH derivatives at increasing concentrations of GSH. Peak No. 1, glutathione; No. 2, cysteine; No. 3, impurity from the derivatizing agent. Other peaks were not identified.

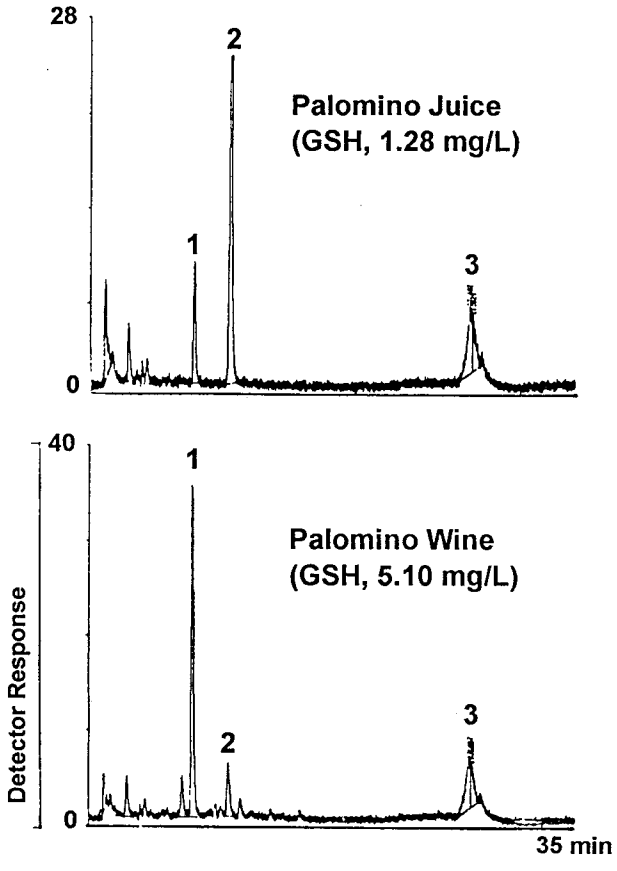

Fig. 4. Analysis of thiols in Palomino grape juice (top) and resulting Palomino wine (bottom). Numbers on Y-axis represent a recorder scale (%). Peak No. 1, glutathione; No. 2, tentatively identified as γ-glutamylcysteine; No. 3, impurity from the derivatizing reagent. No internal standard was added to the samples.

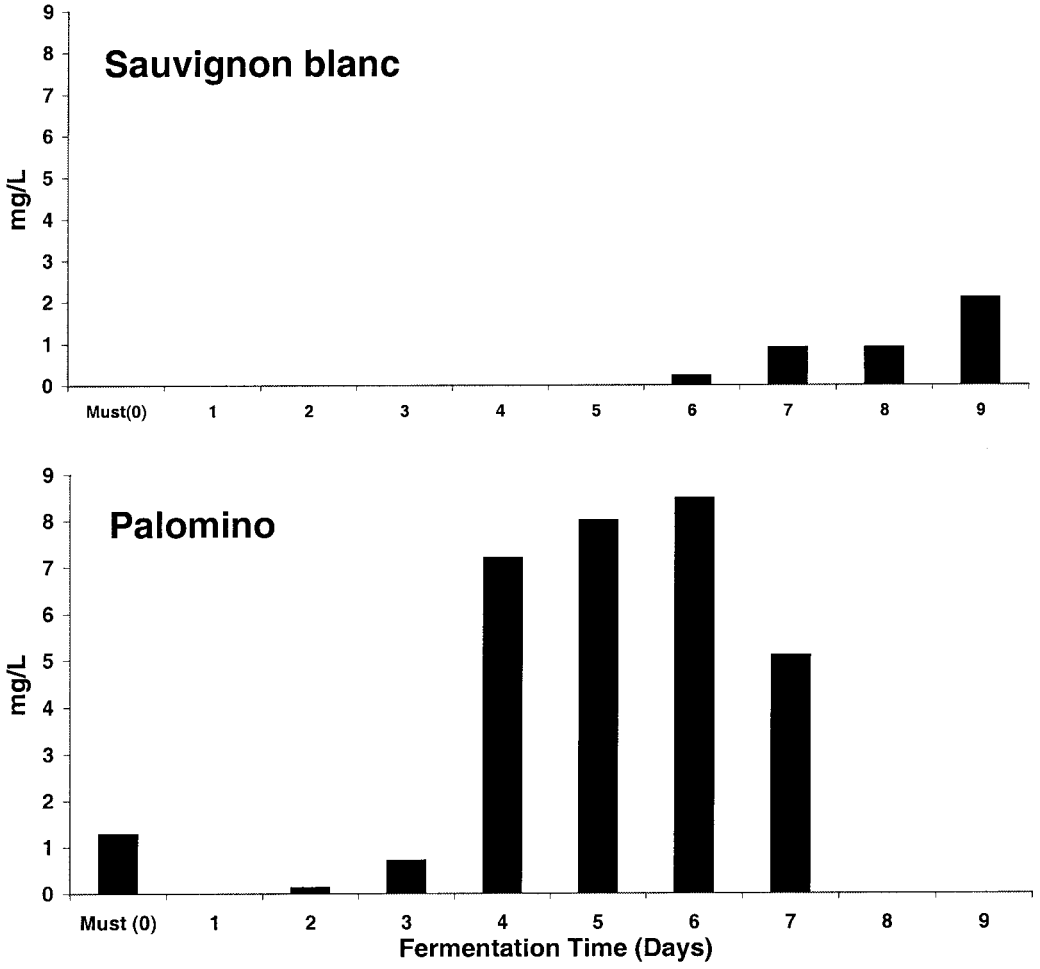

Fig. 5. Concentration of glutathione (mg/l) in starting musts, and throughout fermentation. Top: Sauvignon blanc; bottom, Palomino.

matic hydrolysis product from GSH, and No. 3 is an impurity from the derivatizing agents. Several other unidentified thiol peaks appeared in wine as a result of fermentation.

Before fermentation, the Palomino juice contained 1.28 mg/l GSH, while the Sauvignon blanc had no detectable GSH. As shown in Fig. 5, GSH increased during fermentation in both samples. Following fermentation, 5.1 mg/l GSH was found in the wine vs 2.1 mg/l for the Sauvignon blanc. A similar pattern of GSH increase during fermentation was observed in fermentations of three other varietal grapes (Park et al., 1999). Although the average concentration of GSH in white grapes analyzed under anaerobic conditions was 47 mg/l (Cheynier et al., 1989), musts from crushed grapes, such as these juices, contain very low GSH levels. Oxidative reactions of GSH with hydroxycinnamates occur during grape crushing, yielding the "grape reaction product", 2-*S*-glutathionyl caftaric acid (Singleton, Salgues, Zaya & Trousdale, 1985).

Peak No. 2, tentatively identified as γ-glutamylcysteine, decreased upon fermentation; the concentration was not determined because of unavailability of a standard. No cysteine was found in the juice or wine despite the low detection limit of 2.7 mg/l for cysteine.

4. Conclusions

This in-line pre-column derivatization and HPLC analysis provides sensitive, reproducible and rapid quantification of thiols in grape juice and wines. With a total run time of 35 min, up to 40 samples per day could be run with unattended operation.

Acknowledgements

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