RESOLUTION OIV/OENO 345/2009

DETERMINATION OF GLUTATHIONE IN MUSTS AND WINES BY CAPILLARY ELECTROPHORESIS COUPLED WITH A LASER-INDUCED FLUORESCENCE DETECTOR (LIF)

THE GENERAL ASSEMBLY,

IN VIEW of article 2, paragraph 2 iv of the Agreement of 3 April 2001, establishing the International Organisation of Vine and Wine, following a proposal made by the sub-commission "Methods of Analysis",

HAS HEREBY DECIDED to add the following type IV method to Appendix A of the International Compendium of Methods of Analysis:

Title	Type of method
Determinaiton of glutathione in musts and wines by capillary electrophoresis	IV

1. Scope

This method makes it possible to determine the glutathione content of musts and wines in a concentration range of 0 to 40 mg/L. It uses capillary electrophoresis (CE) associated with fluorimetric detection (LIF).

2. Principle

The method used, which proceeds by capillary electrophoresis, is an adaptation of the method developed by Noctor and Foyer (1998) to determine non-volatile thiols in poplar leaves using HPLC coupled with fluorimetric detection.

The separation of a mixture's solutes by capillary electrophoresis is obtained by differential migration in an electrolyte. The capillary tube is filled with this electrolyte.

The sample to be separated is injected into one end of the capillary tube. As a result of electrical field activity generated by the electrodes immersed in the electrolyte, the solutes separate due to differences in migration speed and are detected near the other end of the capillary tube in the form of peaks. In given operating conditions,

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migration times constitute a criterion for the identification of chemical species and the peak area is proportional to the quantity injected.

3. Products and reagents

3.1. List of products

3.1.1. Glutathione (GSH, > 98 %)

3.1.2. Dithiothreitol (DTT, > 99 %)

3.1.3. Anhydrous monobasic sodium phosphate (NaH2PO4, > 99 %)

3.1.4. Anhydrous dibasic sodium phosphate (Na2HPO4, > 99 %)

3.1.5. 2-(N-cyclohexylamino)ethanesulfonic acid (CHES, > 98 %),

3.1.6. Monobromobimane (MBB, 97 %)

3.1.7. Ethylenediamine tetraacetic acid sodium salt (EDTA, > 99 %)

3.1.8. Sodium hydroxide

3.1.9. Hydrochloric acid (35 %)

3.1.10. Acetonitrile (99.5 %)

3.1.11. Ultra-pure water with a resistance of >18 M□·cm.

3.2. List of solutions

All solutions are homogenised prior to use

3.2.1. Electrophoretic buffer: phosphate buffer, 50 mM, pH 7

This buffer is prepared using two solutions - A and B

3.2.1.1. Solution A: 3 mg of anhydrous monobasic phosphate (3.1.3) taken up by 250 ml ultra-pure water (3.1.11)

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3.2.1.2. Solution B: 3.55 mg of anhydrous dibasic phosphate (3.1.4) taken up by 250 ml ultra-pure water (3.1.11)

The phosphate buffer is obtained by the addition of 40 ml of solution A (3.2.1.1) and 210 ml of solution B (3.2.1.2) and then made up to 500 ml with ultra-pure water (3.1.11). The buffer's pH is then adjusted to 7 using hydrochloric acid (3.1.9).

3.2.2. Monobromobimane solution (MBB) - 50 mM

25 mg of monobromobimane (MBB) (3.1.6) are taken up by 1,850 µl of acetonitrile (3.1.10).
Stored in the dark at -20 °C, this reagent remains stable for three months.

3.2.3. 0.1 M sodium hydroxide solution

0.4 g of sodium hydroxide (3.1.8) are put into a 100-ml volumetric flask and taken up by 100 ml of ultra-pure water (3.1.11).

3.2.4. 5 M sodium hydroxide solution

20 g of sodium hydroxide (3.1.8) are put into a 100-ml volumetric flask and taken up by 100 ml of ultra-pure water (3.1.11).

3.2.5. CHES buffer: 0.5 M, pH 9.3

2.58 g of 2-(N-cyclohexylamino) ethanesulfonic acid (CHES) (3.1.5) are dissolved in approximately 20ml of ultra pure water (3.1.11). The pH buffer is adjusted to 9.3 by the addition of sodium hydroxide 5 M (3.2.4). The volume is then adjusted to 25 ml with ultra pure water (3.1.11). This buffer is divided between the 1.5-ml test tubes (Eppendorf type) with 1 ml per tube. Stored at –20 °C, the CHES aqueous solution may be kept for several months.

3.2.6. Dithiothreitol solution (DTT) - 10 mM

15.4 mg of dithiothreitol (3.1.2) is dissolved in 10 mL of ultra pure water (3.1.11) then this solution is divided in 1.5-ml test tube (Eppendorf type) with 1 ml per tube
Stored at –20 °C, this DTT aqueous solution may be kept several months.

4. Apparatus

4.1. Capillary electrophoresis

Capillary electrophoresis equipped with a hydrostatic-type injector is coupled with a

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laser-induced fluorescence detector with an excitation wavelength similar to the absorption wavelength of the MBB-GSH adduct: e.g.= 390 nm (e.g. Zetalif detector).

4.2. The capillary tube

The total length of the non-grafted silica capillary tube is 120 cm. Its effective length is 105 cm, and its internal diameter is 30 µm.

5. Preparation of samples

The method of determination used consists of the derivatization of the SH functions by the monobromobimane (MBB) (Radkowsky & Kosower, 1986). Samples of musts or non-bottled wines are clarified by centrifugation prior to analysis. Bottled wines are analysed without prior clarification.

Preparation of samples:

In a 1.5-ml test tube (Eppendorf type), put successively:

- 200 µl of the sample,
- 10 µl of the DTT solution (3.2.4) - final concentration of 0.25 mM,
- 145 µl of CHES (3.2.3) - final concentration of 179 mM,
- 50 µl of MBB (3.2.2) - final concentration of 6.2 mM.

After stirring the reagent mixture, the derivatization of thiol functions by the MBB requires a 20-minute incubation period in the dark at ambient temperature. In these analytical conditions, the MBB-SR derivatives thus formed are relatively unstable; CE-LIF determination should be carried out immediately after incubation.

6. Procedure

6.1. Capillary tube preparation

Before being used for the first time and as soon as migration times increase, the capillary tube (4.2) should be treated in the following way:

6.1.1. Rinse with 0.1 M sodium hydroxide (3.2.5) for 3 minutes,

6.1.2. Rinse with ultra-pure water (3.1.12) for 3 minutes,

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6.1.3. Rinse with the electrophoretic phosphate buffer (3.2.1) for 3 minutes.

6.2. Migration conditions

6.2.1. Injection of the sample is of the hydrostatic type; 3 s at 50 kPa.

This is followed by injection of 50 mb electrophoretic buffer (3.2.1) to improve peak resolution (Staking).

6.2.2. Analysis.

A voltage of +30 kV, applied throughout separation, generates a current of 47 µA. These conditions are reached in 20 s. Separation is carried out at a constant temperature of 21 °C.

6.2.3. Rinsing the capillary tube

The capillary tube should be rinsed after each analysis, successively with:

- 0.1M sodium hydroxide (3.2.5) for 3 minutes,
- ultra-pure water (3.1.12) for 3 minutes,
- electrophoretic phosphate buffer (3.2.1) for 3 minutes.

7. Results

At the concentration ultimately used in the sample, the presence of DTT during derivatization makes it possible to stabilise the unstable functions of thiols that have an alkaline pH and are very easily oxidized by quinines produced by phenolic compound auto-oxidation, but does not break the disulphide bonds. Thus, under these analytical conditions, the reduced glutathione content (GSH) found in a wine with or without the addition of 10 mg/l of oxidized glutathione (GSSG) is strictly comparable (Figure 1). This method therefore makes it possible to determine glutathione content in its reduced form alone.

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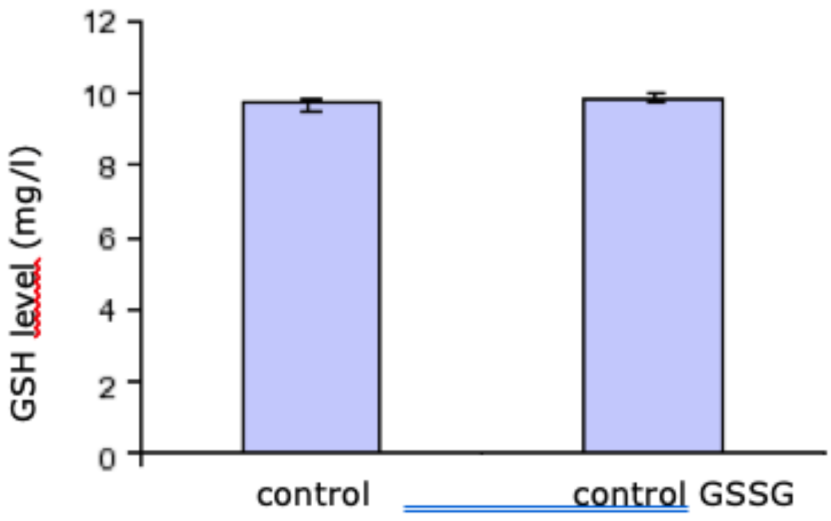


Figure 1: Demonstration of the stability of disulphide bonds according to the conditions of derivatization described. (DTT, ultimately 0.25 mM)

Figure 2 shows the electrophoretic profile of a white grape must sample (Sauvignon) in which cysteine, glutathione, N-acetyl-cysteine and sulphur dioxide are identified. The first peak corresponds to excess reagents (DTT, MBB). The separation of non-volatile thiols takes less than 20 minutes. Only certain peaks could be identified (Figure 2, A) (Newton et al., 1981). These thiols, apart from the sulphur dioxide, are generally present in varying quantities in grapes (Cheynier et al., 1989), fruit and vegetables (Mills et al., 2000).

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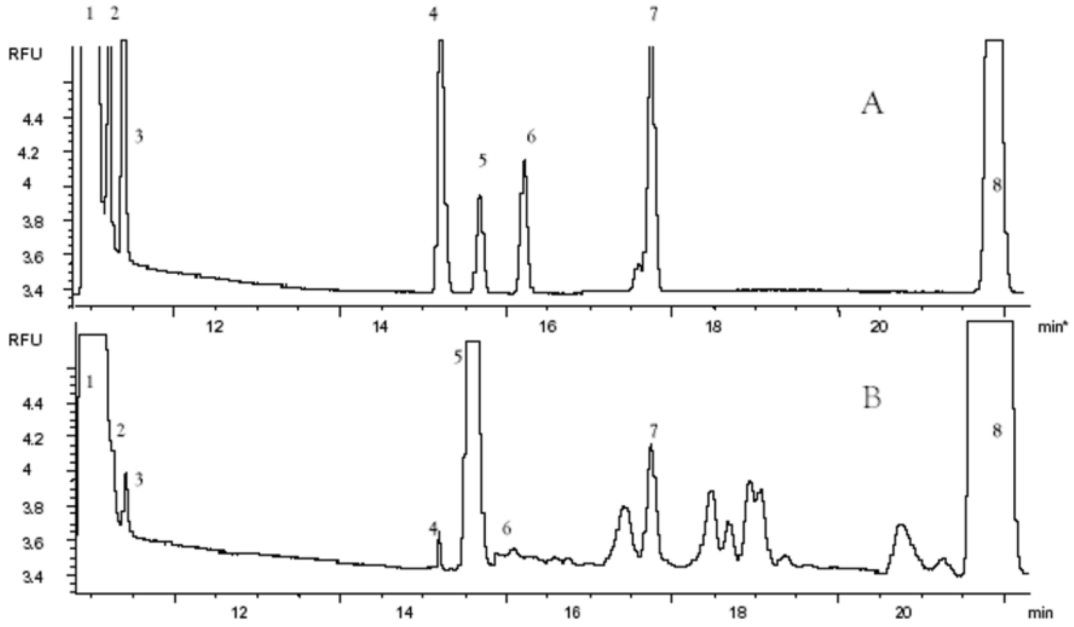


Figure 2: Example of the separation of the known non-volatile thiols in an HCl/EDTA solution (A) 1 and in a grape must (B): DTT; 2: homocysteine; 3: cysteine; 4: Cys-Gly; 5: GSH; 6: g Glu-Cys; ,7: NAC; 8: SO_2 .

In these analytical conditions, MBB-RS adduct retention times are as follows: MBB-homocysteine 10.40 mins; MBB-cysteine 10.65 mins, MBB-GSH 14.14 mins; MBB-NAC 15.41mins; MBB-SO_2 18.58mins.

8. Characteristics of the method

Certain internal elements of validation were determined, but do not constitute formal validation according to the protocol for the design, conducts and interpretation of methods of analysis performance studies (OIV 6/2000).

Wine is used as a matrix to produce calibration curves and repeatability tests for each compound. Each concentration is calculated based on the average of three determinations obtained by using the right of the calibration curb regression. Results are expressed in mg/L.

Linear regressions and correlation coefficients are calculated according to the least squares method. The stock solutions of the various thiols are produced from an HCl/EDTA solution, allowing them to be stored at +6 °C for several days with no loss. Successive dilutions of these solutions allow the threshold limits for detection in wine

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to be estimated, for a signal-to-noise ratio of three of more.

The linearity spectrum varies according to thiols (Table 1).

Table 1: Linearity spectrum, linear regression properties for each thiol in solutions prepared in exactly the same way as that of the glutathione.

	Linearity spectrum	Linear regression	Correlation coefficient
Homocysteine	0 - 15 mg/l	Y= 0.459X – 0.231	0.9987
Cysteine	0 - 15 mg/l	Y = 0.374X – 0.131	0.9979
Glutathione	0 - 40 mg/l	Y = 0.583X – 0.948	0.9966
N-acetyl-cysteine	0 - 10 mg/l	Y = 0.256X – 0.085	0.9982

These analytical conditions make it possible to eliminate interference caused by MBB hydrolysis products, unlike the reported findings of other works (Ivanov et al., 2000).

The method's repeatability is calculated on the basis of ten analyses of the same sample of wine. For a thiol concentration of 10 mg/l, the coefficient of variation is 6.0 % for the glutathione; besides this, it is 3.2 % for the homocysteine, 4.8 % for the cysteine and 6.4 % for the N-acetyl-cysteine.

The limit for detecting glutathione is 20 µg/l and the quantification limit is 60 µg/l.

9. Bibliography

1. Noctor, G. and C. Foyer, 1998. Simultaneous measurement of foliar glutathione, gamma-glutamylcysteine, and amino acids by high-performance liquid chromatography: comparison with two other assay methods for glutathione, Analytical Biochemistry, 264, 98-110.
2. Kosower, N.S., Kosower E. M., Newton G. L.,and Ranney H. M., 1979. Bimane fluorescent labels: Labeling of normal human red cells under physiological conditions. Proc. Natl. Acad. Sci., 76 (7), 3382-3386.
3. Newton, G.L., R. Dorian, and R.C. Fahey, Analysis of biological thiols: derivatisation with monobromobimane and separation by reverse-phase high-performance liquid

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chromatography. Anal. Biochem., 1981. 114: p. 383-387.

4. Cheynier, V., J.M. Souquet, and M. Moutounet, 1989. Glutathione content and glutathione to
5. hydroxycinnamique acid ration in Vitis vinifera grapes and musts. Am. J.Enol.Vitic,. 40 (4), 320-324.
6. Mills, B.J., Stinson C. T., Liu M. C. and Lang C. A., 1997. Glutathione and cyst(e)ine profiles of vegetables using high performance liquid chromatography with dual electrochemical detection. Journal of food composition and analysis, 10, 90-101.
7. Ivanov, A.R., I.V. Nazimov, and L. Baratova, 2000. Determination of biologically active low molecular mass thiols in human blood. Journal of Chromatogr. A,. 895, 167-171.

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