COEI-1-LEVGLU Inactivated yeasts with guaranteed glutathione levels

1. Object, origin and scope of application

These inactivated dry yeasts (IDY) with guaranteed glutathione levels are characterised by higher levels of reduced glutathione than those contained in standard inactivated yeasts. They are used to limit oxidation phenomena in musts and wines. The presence of reduced glutathione (GSH) may be accompanied by that of its precursors, cysteine and – in particular – gamma-glutamylcysteine.

Like classic inactivated dry yeasts, they also provide nutrients for yeasts at the start of and during alcoholic fermentation. They may help to reduce ochratoxin-A levels in wines during maturing and clarification operations[i] (Resolution OIV-OENO 459-2013).

They are derived from *Saccharomyces* and/or *non-Saccharomyces* species' biomass, whose cultivation is managed so as to increase the natural production of reduced glutathione (GSH). They are therefore derived from pure cultures without any subsequent addition of glutathione or of cysteine and gamma–glutamyl-cysteine to the final product, what is attested by a ratio between gamma-glutamyl-cysteine and GSH that must be superior to 20%.

They are inactivated by heat and/or by pH modification. They may have been subjected to the start of natural autolysis under the action of endogenous enzymes. No antibiotics or other compounds are added in the process other than those necessary for yeast growth.

When the inactivated yeasts come from genetically engineered yeasts, these must have received the preliminary authorisation of the relevant authorities.[1]

2. Labelling

The following must appear on the label:

- the name of the genus and species of the inactivated yeasts with guaranteed glutathione levels,
- the minimum content , expressed in mg/g of inactivated dry yeasts (IDY), of reduced glutathione
- the maximum content, expressed in mg/g of inactivated dry yeasts (IDY), of cysteine
- the maximum content, expressed in mg/g of inactivated dry yeasts (IDY), of gamma-glutamyl-cysteine,
- the organic nitrogen content,

- any additives,
- the instructions for use,
- the batch number as well as the expiry date and storage conditions such as temperature, humidity and aeration conditions,
- the indication that the inactivated yeasts come from genetically engineered yeasts, and the modified character if this is the case.

3. Characteristics

They are most often in the form of granules, powder or flakes, of light yellow to yellow ochre, with a smell characteristic of yeast. Inactivated yeasts with guaranteed GSH levels are partially soluble in water, with the insoluble part being greater or equal to 60% m/m of the dry matter.

4. Limits and trial methods

4.1. Oxidised glutathione (GSSG) content

The level of oxidised form of glutathione, glutathione disulphide (GSSG) that is the only identified form according to the state of our knowledge, is measured by the HPLC method described in Annex 1.

4.1.1. Preparation of the solution for testing

Precisely weigh 2 g IDY and place in a 20-mL centrifuge tube, add 1 mL glass beads of 425-600 microns and 4 mL pH 7.5 phosphate buffer solution.

Vortex for 20 min at 4°C then centrifuge a minimum of 12,000 g for 20 min at 4°C.

The supernatant is the solution for testing, which is to be kept in the dark at 4°C for 4 hrs maximum before determination.

The ratio between reduced gluthatione and oxidised glutathione should be higher than3.

4.2. Reduced glutathione (GSH), Cysteine and gamma-glutamylcysteine content

The reduced glutathione, cysteine and gamma-glutamylcysteine levels are measured by the HPLC method after derivatisation described in Annex 4.

The reduced glutathione content should be greater than 1%, or 10 mg/g IDY

The endogenous cysteine content should be lower than 0.3%, or 3 mg/g IDY,

the gamma-glutamylcysteine content should be lower than 1%, or 10 mg/g IDY.

4.3. Nitrogen content

4.3.1. The total nitrogen content, expressed as element N, is less than 10% of the dry matter, according to the method of analysis described in Chapter II of the *International Oenological Codex*, being referred to as N_t

4.3.2. The ammoniacal nitrogen content, expressed as element N, must be less than 0.5% of the dry matter and is determined according to the method of analysis described in Annex 2, being referred to as N_a

4.3.3. The organic nitrogen content is obtained by the difference between the total nitrogen content and the ammoniacal nitrogen content:

Organic nitrogen = N_t-N_a

4.3.4. The small peptide and free and soluble amino acid content should be less than 10% of the dry matter in glycine equivalent, according to the DNFB method described in Annex 3, or 1.9% of the dry matter expressed as element N.

4.4. Humidity

This is measured by the 5 g loss in product weight, dried at 105 °C to constant weight (around 3 hours).The maximum content should be less than 7%.

4.5. Lead

Proceed with an analysis according to the method that appears in Chapter II of the *International Oenological Codex*.

The content should be less than 2 mg/kg of the dry matter.

4.6. Mercury

Proceed with an analysis according to the method that appears in Chapter II of the *International Oenological Codex*.

The content should be less than 1 mg/kg of the dry matter.

4.7. Arsenic

Proceed with an analysis according to the method that appears in Chapter II of the *International Oenological Codex*.

The content should be less than 3 mg/kg of the dry matter.

4.8. Cadmium

Proceed with an analysis according to the method that appears in Chapter II of the *International Oenological Codex*.

The content should be less than 1 mg/kg of the dry matter.

4.9. Viable yeasts

Proceed with counting according to the method that appears in Chapter II of the *International Oenological Codex*.

The number should be less than or equal to 10^2 CFU/g of the dry matter.

4.10. Mould

Proceed with counting according to the method that appears in Chapter II of the

International Oenological Codex.

The number should be less than 10^3 CFU/g of the dry matter.

4.11. Lactic acid bacteria

Proceed with counting according to the method that appears in Chapter II of the *International Oenological Codex*.

The number should be less than 10^3 CFU/g of the dry matter.

4.12. Acetic bacteria

Proceed with counting according to the method that appears in Chapter II of the *International Oenological Codex*.

The number should be less than 10^3 CFU/g of the dry matter.

4.13. Salmonella

Proceed with counting according to the method that appears in Chapter II of the *International Oenological Codex*.

Absence should be checked on a 25 g sample of the dry matter.

4.14. Escherichia coli

Proceed with counting according to the method that appears in Chapter II of the *International Oenological Codex*.

Absence should be checked on a 1 g sample of the dry matter.

4.15. Staphylococci

Proceed with counting according to the method that appears in Chapter II of the *International Oenological Codex*.

Absence should be checked on a 1 g sample of the dry matter.

4.16. Coliforms

Proceed with counting according to the method that appears in Chapter II of the *International Oenological Codex*.

The number should be less than 10^2 CFU/g of the dry matter.

5. Additives

These should be compliant with the regulations in force.

6. Preservation

Do not store in open packaging. The inactivated yeasts with guaranteed glutathione levels should always be kept in sealed sachets without air contact. Store in a cool, dry place. Follow the manufacturer's instructions at all times. Storage under

unappropriated conditions might lead to the decrease of the reduced glutathione content.

Annex 1: Determination of reduced and oxidised glutathione by HPLC

This determination is carried out according to the method for the determination of glutathione in pharmaceutical preparations by Soliman *et al.* (2014).

1. Scope of application

This method makes it possible to determine the reduced glutathione and oxidised glutathione or glutathione disulphide (GSSG) levels within a concentration range of 0-100 mg/L of preparation for analysis.

2. Principle

The method used employs high-performance liquid chromatography according to the reverse-phase principle (column C18) with detection by spectrophotometry using diode-array apparatus of 200-400 nm.

3. Products and reagents

3.1. List of products

3.1.1. Glutathione (GSH, > 98%)

3.1.2. Methanol (HPLC-grade purity)

3.1.3. Formic acid (purity > 98%)

3.1.4. Ultra-pure water with resistivity of >18 M□.cm at 25°C temperature.

3.2. Mobile phase

The mobile phase is constituted of ultra-pure water (3.1.4) containing 0.1% of the formic-acid mixture (3.1.3) and methanol (3.1.2) in proportions of 90:10, v/v.

4. Equipment

4.1. High-performance liquid chromatography apparatus
4.2. Diode-array spectrophotometer
4.3. Data-acquisition apparatus
4.4. Octadecyl-type column of dimensions 150 mm x 2 mm and with a 3-µm diameter (by way of example)
4.5. 230-µL Loop injector
4.6. System for degassing of solvents (ultrasonic)
4.7. System for filtration of samples on a membrane with pores of 0.45 µm in diameter.

5. Preparation of samples

5.1. The sample containing the glutathione to be determined is prepared by dilution of the solution for testing (point 4.1.1 of the monograph) in the mobile phase (3.2) in order to obtain a final concentration of around 20 mg/L.

5.2. The samples are filtered on a membrane (4.7) before injection.

6. Procedure

Analysis is conducted at room temperature, in isocratic mode with a mobile-phase flow rate of 0.5 mL/min.

Detection is carried out in "scan" mode at 200-400 nm.

7. Results

Under these analytical conditions, reduced glutathione (GSH) is well separated from oxidised glutathione (GSSG). This method consequently allows both forms of glutathione to be determined.

Under these analytical conditions, the retention time of glutathione is 7.5 min and that of oxidised glutathione is 9.5 min.

8. Method characteristics

Each concentration is calculated by averaging the three determinations obtained by using the regression line of the calibration curve. The results are expressed in mg/L.

The linear regression and correlation coefficient are calculated according to the least-squares method.

Linearity

- The linear range is 0-100 mg/L and the R correlation coefficient = 0.9998.

Precision

- The method precision was evaluated based on 3 analyses of glutathione at 1.0, 50.0 and 100.0 mg/L, conducted within the same day and also on 3 different days.

Table 1: Characteristics of the method for the determination of reduced glutathione based on the recovery rates

	Daily precision	Precision over 3 days

GSH concentration (mg/L)	% recovery, SD	% CV	% recovery, SD	% CV
1	99.88 ± 0.68	0.68	99.76 ±1.89	1.89
50	100.04 ± 0.39	0.39	100.09 ± 0.73	0.73
100	99.93 ± 0.57	0.57	99.85 ± 0.86	0.86

Scope of application

- According to the determinations carried out, the method is applicable for concentrations of 0-100 mg/L.

The limits of detection and quantification of glutathione established according to the guidelines of the International Conference on Harmonisation (ICH) (3.3 and 10 times the standard deviation of the blank [7 analyses] divided by the slope of the calibration curve) are 20 µg/L (LoD) and 68 µg/L (LoQ) respectively.

9. Bibliography

- Soliman, R. M., Hadad, G. M., Abdel Salam, R.A., Mesbah, M. K., 'Quantitative determination of glutathione in presence of its degradant in a pharmaceutical preparation using HPLC-DAD and identification by LC-ESI-MS', *J. Liquid Chromatography and related technologies*, 37, 2014, pp. 548-559.

Annex 2: Determination of ammoniacal nitrogen

1. Reagents

1.1. Potassium chloride (0.5 M KCl)

Dissolve 18.64 g KCl in 500 mL pure, demineralised water.

1.2. 30% Sodium hydroxide

Place 30 g sodium hydroxide in a 100-mL flask, add 70 mL pure, demineralised water, stir until dissolved and make up to 100 mL.

1.3. 4% Boric acid (R)

See R part II of the *International Oenological Codex*.

1.4. M Hydrochloric acid for titration (solution ready for market use)

1.5. Mixed indicator of methyl red and methylene blue

See R part II of the *International Oenological Codex*.

2. Equipment

2.1. Laboratory glassware

2.2. Steam distillation apparatus as described in Chapter II of the *International Oenological Codex* for the determination of total nitrogen.

3. Determination

3.1. Place 1 g of the dry matter of the inactivated yeasts in 100 mL 0.5 M KCl (1.1) and mix for 20-30 min.

3.2. Introduce the 100 mL into the steam distillation apparatus (2.2) with 50 mL 30% sodium hydroxide (R).

3.3. Distil by collecting 250 mL in a conical flask containing 5 mL 4% boric acid (1.3), 10 mL water and 2-3 drops of mixed indicator of methyl red and methylene blue (1.5).

Titrate the distillate using 0.1 M hydrochloric acid (1.4) up to the purple-pink bend of the indicator.

1 mL hydrochloric acid solution corresponds to 1.4 mg nitrogen (N).

Where *n* is the number of mL poured:

100 g IDY contain 0.14 *n* g ammoniacal nitrogen expressed as element N, namely N_a.

Annex 3:Amino nitrogen method

1. Introduction

This method makes it possible to quickly determine the amino nitrogen in a biological solution compared with a calibration range produced with glycine solution.

2. Scope of application

Oenological products of plant or animal origin.

3. Definition

Dinitrofluorobenzene or DNFB reacts with the free NH_2 groups contained in the amino acids in order to give a compound with a bright yellow colour determined by 420-nm colorimetry. The reaction takes place at a pH > 9.3.

4. Reagents and products

4.1. Reagents:

4.1.1. borax or sodium tetraborate,

4.1.2. dinitrofluorobenzene (be careful of hazards while handling DNB),

4.1.3. 10 M hydrochloric acid,

4.1.4. glycine, purity ≥ 98%,

4.1.5. ethanol 95% vol.

5. Apparatus

5.1. Haemolysis tubes,
5.2. micropipettes,
5.3. spectrophotometer for measurements in the visible region,
5.4. 60 °C water bath.

6. Preparation of samples

1. Prepare a 5% sodium tetraborate solution in pure water.
2. Prepare a DNFB solution: introduce 130 µL DNFB in 10 mL ethanol of 95% vol.,
3. Prepare a 2 M hydrochloric acid solution,
4. Create a calibration range from a 2 g/L glycine stock solution (M=75.07 g) e.g. 0.50 mg/L, 100 mg/L, 200 mg/L, 500 mg/L,
5. Prepare a 2 g/L suspension of the product to be determined, centrifuge for 30 min and recover the supernatant.

7. Procedure

Introduce the following into a haemolysis tube:

- 380 µL 5% borax (6.1),
- 20 µL sample to be determined (6.5)
- 20 µL DNFB solution (6.2),

Do the same for the glycine range,
Mix and place the water bath at 60°C for 30 min (5.4),
Add 3 mL 2M HCl (6.3),
Mix and read the specific absorbance at 420 nm for the sample (5.3),
Produce a calibration range with the glycine range (6.4).

8. Results

Record the absorbance value of the sample at 420 nm on the calibration curve. The results are expressed in g glycine/L.

Annex 4: Determination of reduced glutathione, cysteine and gamma-glutamylcysteine by HPLC after derivatisation

Preambule

The principle is to determine, by HPLC/UPLC-UV using a reverse-phase column, amino acids and thiol peptides after derivatisation of this function. This method is suitable for complex matrices of yeasts and yeast derivatives.

1. Scope of application

This method allows for the determination of reduced glutathione (GSH), cysteine (Cys) and gamma-glutamylcysteine (GluCys) within the following concentration ranges:

- 2-24 mg/L for the GSH and GluCys compounds,
- 0.5-6 mg/L for Cys.

2. Principle

The method used employs high-performance liquid chromatography according to the reverse-phase principle (column C18) with detection by spectrophotometry at 320 nm.

3. Products and reagents

3.1. Products

3.1.1. GSH: glutathione, CAS no. 70-18-8 (purity > 98%)

3.1.2. Cys.HCl.$H_2$0: L-cysteine hydrochloride monohydrate, CAS no. 7048-04-6 (purity > 98%)

3.1.3. GluCys: □-L-glutamyl-L-cysteine, CAS no. 636-58-8 (purity > 80%)

3.1.4. Sodium dihydrogen phosphate (NaH2PO4·H2O), pure

3.1.5. Sodium acetate anhydrous, pure

3.1.6. 17.4 M Acetic acid, pure

3.1.7. Methanol (HPLC-grade purity)

3.1.8. Concentrated phosphoric acid (purity > 98%)

3.1.9. Ultra-pure water of resistivity >18 M.cm at a temperature of 25°C

3.1.10. Acetonitrile, pure

3.1.11. 2,2'-Dithiobis(5-nitropyridine) (DNTP), CAS no. 2127-10-8 (purity > 96%)

3.1.12. Concentrated trichloroacetic acid solution (25-30%)

3.2. Acetate buffer (used for derivatisation)

- Weigh 8.1 g sodium acetate (3.1.5), dissolve into 100 mL ultra-pure water (3.1.9),
- adjust the pH to 6.3 with acetic acid (3.1.6) (around 100-200 µL),
- make up to 1 L with ultra-pure water (3.1.9).

3. 2,2'-Dithiobis(5-nitropyridine) (DNTP) reagent (to be prepared just before use)

- Weigh 30 mg DNTP (3.1.11) and dissolve in 10 mL acetonitrile (3.1.10).

4. Trichloroacetic acid at 5.7%

- Dissolve 19 g trichloroacetic acid at 30% (3.1.12) in 100 mL ultra-pure water (3.1.9).

5. Mobile phase

- Eluent A: weigh 3.4g $NaH_2PO_4 \cdot H_2O$ (3.1.4), dissolve in 898 g ultra-pure water (3.1.9), add 79 g methanol (3.1.7), and adjust the pH from 4.45 to 2.5 by addition of concentrated phosphoric acid (3.1.8; around 0.8-1 mL).
- Eluent B: methanol (3.1.7).

4. Equipment

4.1. High-performance liquid chromatography apparatus
4.2. Spectrophotometer with detection at 320 nm
4.3. Data-acquisition apparatus
4.4. Otadecyl-type column with dimensions of 250 mm x 4.6 mm with a phase diameter of 5 µm (e.g. RP Supelcosil ABZ+Plus; Waters XTerra RP18 or equivalent)
4.5. Loop injector
4.6. System of degassing of solvents (ultrasound)
4.7. Sample filtration system on a membrane with 0.45- µm pore diameter
4.8. Magnetic stirrer
4.9. Centrifuge
4.10. pH meter
4.11. Everyday laboratory glassware

5. Preparation of samples

5.1. Preparation of standards
5.1.1. GSH solution at ~400 mg/L

- In a 200-mL flask, dissolve ~80 mg GSH (3.1.1) weighed exactly, and make up to 200 mL using ultra-pure water (3.1.9).

2. GluCys solution at ~400 mg/L

- In a 50-mL flask, dissolve ~20 mg GluCys (3.1.3) weighed exactly, and make up to 50 mL using ultra-pure water (3.1.9).

3. Cys.HCl.$H_2$0 solution at ~100 mg/L

- In a 100-mL flask, dissolve ~130 mg Cys,HCl.H_2O (3.1.2) weighed exactly, make up to 100 mL using ultra-pure water (3.1.9), then dilute to 1:10 with ultra-pure water (3.1.9).

2. Preparation of samples

The test portion (TP) of the sample should be adapted so that the concentration is within the calibration range, i.e. between 2 and 24 mg/L GSH. For inactivated dry yeast (IDY) with guaranteed GSH levels, take a sample in advance according to the following protocol:

- weigh ~1g IDY exactly, add 17.5 mL trichloroacetic acid at 5.7% (3.4),
- mix for 20 min at room temperature (4.8),
- adjust to 50 mL (=V) with ultra-pure water (3.1.9),
- centrifuge for 10 min at 5500 rpm (4.9).

6. Derivatisation

- Carry out in test tubes based on the preparations in 5.1, according to the following table.
- Mix by inverting the tubes.

The reaction is complete in 5 minutes. The different solutions are analysed by HPLC after filtration (4.7).

							In duplicate
		Standard 1 GSH and GluCys: 2 mg/L Cys: 0.5 mg/L	**Standard 2** GSH and GluCys: 4 mg/L Cys: 1 mg/L	**Standard 3** GSH and GluCys: 8 mg/L Cys: 2 mg/L	**Standard 4** GSH and GluCys: 16 mg/L Cys: 4 mg/L	**Standard 5** GSH and GluCys: 24 mg/L Cys: 12 mg/L	**Test**
Acetate buffer (mL)		8.85	8.7	8.4	7.8	7.2	8
Standard solution (µl)	**GSH (400 mg/L)**	50	100	200	400	600	
	Cys (100 mg/L)	50	100	200	400	600	
	GluCys (400 mg/L)	50	100	200	400	600	
Sample (µl)							1000
DNTP (µl)		1000					

							In duplicate
		Standard 1 GSH and GluCys: 2 mg/L Cys: 0.5 mg/L	**Standard 2** GSH and GluCys: 4 mg/L Cys: 1 mg/L	**Standard 3** GSH and GluCys: 8 mg/L Cys: 2 mg/L	**Standard 4** GSH and GluCys: 16 mg/L Cys: 4 mg/L	**Standard 5** GSH and GluCys: 24 mg/L Cys: 12 mg/L	**Test**
Acetate buffer (mL)		8.85	8.7	8.4	7.8	7.2	8
Standard solution (µl)	**GSH (400 mg/L)**	50	100	200	400	600	
	Cys (100 mg/L)	50	100	200	400	600	
	GluCys (400 mg/L)	50	100	200	400	600	
Sample (µl)							1000
DNTP (µl)		1000					

Important note: Consider adapting the test portion according to the colouration. Verify that the tests fall within the range.

7. Chromatographic conditions

Column temperature: 30 °C

Sample temperature: 5 °C

Mobile-phase flow rate: 1 mL/min

Injection volume: 5 µL

Pressure: 140-175-10^3 hPa (around 2000-2500 psi)

Detection: 320 nm

Duration of analysis: 34 min

Equilibration limit at the end of analysis: 10 min

Rinsing: water

Storage: water / methanol 80:20 v/v

Time after injection (min)	% eluent A	% eluent B
	100	0
1	100	0
23	40	60
28	0	100
32	0	100
34	100	0

7. Calibration curves

For each analyte, establish calibration curves C (mg/L) = f[A] considering the following:

- the concentrations in mg/L for Cys, GSH and GluCys,
- the factor of dilution,
- the areas obtained.

Important note: For Cys, take into consideration the HCl: PE (g/L) * Mpure Cys (121.16 g/mol) / MCys,HCl.H_2O (175.63 g/mol).

Calculations of concentrations:

In terms of dry matter (DM):

$$g/100g\ DM = \frac{Area}{slope} \times \frac{V(=50ml)}{PE(g)} \times \frac{1}{10xDM}$$

In terms of the product as it is:

$$g/100g = \frac{Area}{slope} \times \frac{V(=50ml)}{PE(g)} \times \frac{1}{1000}$$

8. Bibliography

- Rahman et al., Nature Protocols 1, 2007, pp. 3159-3165.
- Katrusiac et al., 'Pre-column derivatization high-performance liquid chromatographic method for determination of cysteine, cysteinyl–glycine,

homocysteine and glutathione in plasma and cell extracts', Journal of chromatography B: Biomedical Sciences and Applications, Vol. 758, No. 2, 2001, pp. 207-212.

- Raju N. Appala et al., 'A Simple HPLC-UV Method for the Determination of Glutathione in PC-12 Cells', Scientifica, Vol. 2016 (2016).

[1] *Code of good vitivinicultural practices in order to minimise the presence of OTA in vine-based products.*