OIV-MA-AS311-07 Joint determination of the glucose and fructose content in wines by differential ph-metry

Type III method

1. Scope

This method is applicable to the analysis of glucose and fructose in wines between 0 and 60 g/L (average level) or 50 and 270 g/L (high level).

2. Principle

The joint determination of glucose and fructose content by differential pH-metry consists in the phosphorylation of the glucose and fructose by hexokinase. The H^+ ions generated stoechiometrically in relation to the quantities of glucose and fructose are then quantified.

3. Reactions

The glucose and fructose present are phosphorylated by adenosine triphosphate (ATP) during an enzymatic reaction catalysed by hexokinase (HK) (EC. 2.7.1.1)

$$glucose + ATP \xrightarrow{HK} glucose - 6 - phosphate + ADP + H^+$$

$$fructose + ATP \xrightarrow{HK} fructose - 6 - phosphate + ADP + H^+$$

4. Reagents

4.1. Demineralised Water (18 M□) or bi-distilled

4.2. 2-Amino-2-(hydroxymethyl)propane-1,3-diol (TRIS) purity ≥ 99%

4.3. Disodic adenosine triphosphate (ATP, 2Na) purity ≥ 99%

4.4. Trisodium phosphate with twelve water molecules (Na3PO4·12H2O) purity ≥ 99%

4.5. Sodium hydroxide (NaOH) purity ≥ 98%

4.6. Magnesium chloride with six water molecules (Mg$Cl_2 \cdot 6H_2O$ purity ≥ 99%

4.7. Triton X 100

4.8. Potassium chloride (KCl) purity ≥ 99%

4.9. 2-Bromo-2-nitropropane-1,3-diol (Bronopol) ($C_3H_6BrNO_4$)

4.10. Hexokinase (EC. 2.7.1.1) 1 mg ≅145 U (e.g. Hofmann La Roche, Mannheim, Germany ref. Hexo-70-1351)

4.11. Glycerol purity ≥ 98%

4.12. Glucose purity ≥ 99%

4.13. Reaction buffer pH 8.0 commercial or prepared according to the following method:

In a graduated 100-ml flask (5.2) pour roughly 70 ml (5.3) of water (4.1), and continuously stir (5.5). Add 0.242 g ± 0.001 g (5.4) of TRIS (4.2), 0.787 g ± 0.001 g (5.4) of ATP (4.3), 0.494 g ± 0.001 g (5.4) of sodium phosphate (4.4), 0.009 mg ± 0.001 g (5.4) of sodium hydroxide (4.5), 0.203 g ± 0.001 g (5.4) of magnesium chloride (4.6), 2.000 ± 0.001 g (5.4) of Triton X 100 (4.7), 0.820 g ± 0.001 g (5.4) of potassium chloride (4.8) and 0.010 ± 0.001 g (4.9) of bronopol. Adjust to volume with water (4.1). The final pH must be 8.0 ± 0.1 (5.6), otherwise adjust it with sodium hydroxide or hydrochloric acid. The buffer thus prepared is stable for two months at 4°C.

4.14. Enzyme solution commercial or prepared according to the following method: Using a graduated pipette (5.7) place 5 ml of glycerol (4.11) into a graduated 10-ml flask, adjust to volume with water (4.1) and homogenize. Dissolve 20 mg ± 1 mg (5.4) of hexokinase (4.10) and 5 mg of Bronopol (4.9) in 10 ml of the glycerol solution. The activity of the enzyme solution must be 300 U ± 50 U per ml for the hexokinase. The enzyme solution is stable for 6 months at 4°C.

4.15. Preparation of the calibration solution (average level) if the supposed content is less than 50 g/L of glucose + fructose)

Place 3.60 g ± 0.01 g (5.4) of glucose (4.12) (desiccated 12 hours beforehand at 40 °C until constant weight), 0.745 g ± 0.001 g (5.4) of potassium chloride (4.8) and 0.010 g ± 0.001 g of bronopol (4.9) in a graduated 100-ml flask (5.2). Add water (4.1). Fully homogenize (5.5). Adjust to volume with water (4.1) after removing the magnetic bar. The final concentration is 36 g/L of glucose. The solution is stable for 6 months at 4 °C.

4.16. Preparation of the calibration solution (high level) if the supposed content is above 50 g/L of glucose + fructose)

Place 18.0 g ± 0.01 g (5.4) of glucose (4.12) (desiccated 12 hours beforehand at 40 °C until constant weight), 0.745 g ± 0.001 g (5.4) of potassium chloride (4.8) and 0.010 g ± 0.001 g of bronopol (4.9) in a graduated 100-ml flask (5.2). Add water (4.1). Fully homogenize (5.5). Adjust to volume with water (4.1) after removing the magnetic bar. The final concentration is 180 g/L of glucose. The solution is stable for 6 months at 4°C.

5. Apparatus

5.1. Differential pH-metry apparatus (EUROCHEM CL 10 plus, Microlab EFA or

equivalent) see appendix A

5.2. Graduated 100-ml flask, class A

5.3. Graduated 100-ml test-tube with sole

5.4. Precision balance to weigh within 1 mg

5.5. Magnetic stirrer and magnetic Teflon bar

5.6. pH-meter

5.7. Graduated 3-mL, 5-mL pipettes, class A

5.8. Graduated 10-ml flask, class A

5.9. Automatic syringe pipettes, 25 and 50 µL

6. Preparation of samples

The samples should not be too charged with suspended matter; in the contrary case, centrifuge or filter them. Sparkling wines must be degassed.

7. Procedure

The operator must respect the instructions for use of the equipment (5.1). Before any use, the instrument must be stabilized in temperature. The circuits must be rinsed with the buffer solution (4.13) after cleaning, if required.

7.1. Determination of the blank (determination of the enzyme signal)

Fill the electrode compartments (EL_1 and EL_2) of the differential pH-meter (5.1) with the buffer solution (4.13); the potential difference between the two electrodes (D_1) must range between $\pm$ 150 mpH;

Add 24 µL of enzyme solution (4.14) to the reaction vessel (using the micropipette 5.9 or the preparer) and fill electrode EL_2;

Measure the potential difference (D_2) between the two electrodes;

Calculate the difference in pH, ΔpH_0 for the blank using the following formula:

$$\Delta pH_0 = D_2 - D_1$$

where

ΔpH_0 = the difference in pH between two measurements for the blank;

D_1 = the value of the difference in pH between the two electrodes filled with the buffer solution;

D_2 = the value of the difference in pH between the two electrodes, one of which is filled with the buffer solution and the other with the buffer solution and enzyme solution.

The value of ΔpH_o is used to check the state of the electrodes during titration as well as their possible drift over time; it must lie between −30 and 0 mpH and $\leq$ 1.5 mpH between two consecutive readings. If not, check the quality of the buffer pH and the cleanliness of the hydraulic system and electrodes, clean if necessary and then repeat the blank.

7.2 Calibration

7.2.1 Average level

Fill the electrode compartments (EL_1 and EL_2) with the buffer solution (4.13);

Add 25 µL (with the micropipette 5.9 or the preparer) of the standard glucose solution (4.15) to the reaction vessel;

Fill the electrodes EL_1 and EL_2 with the buffer + standard solution;

Measure the potential difference (D_3) between the two electrodes;

Add 24 µL of enzyme solution (4.14) and fill electrode EL_2 with the buffer + standard solution + enzyme;

After the time necessary for the enzymatic reaction, measure the potential difference (D_4) between the two electrodes;

Calculate the difference in pH, ΔpH_C for the calibration sample using the following formula:

$$\Delta pH_C = (D_4 - D_3) - \Delta pH_o$$

where

ΔpH_C = the difference between two measurements D_3 and D_4 for the calibration sample minus the difference obtained for the blank;

D_3 = the value of the difference in pH between the two electrodes filled with the reference buffer/solution mixture;

D_4 = the value of the difference in pH between the two electrodes, one of which is filled with the reference buffer/solution and the other with the buffer/ enzyme / reference solution.

Calculate the slope of the calibration line:

$$s = C_u / \Delta pH_c$$

where

- C_u is the concentration of glucose in the standard solution expressed in g/L.

Check the validity of the calibration by analysing 25 µL of standard solution (ML) of glucose (4.15) according to the procedure (7.3). The result must range between ± 2% of the reference value. If not, repeat the calibration procedure.

7.2. High level

Fill the electrode compartments (EL_1 and EL_2) with the buffer (4.13);

Add 10 µL (with the micropipette 5.9 or the preparer) of standard solution (HL) of glucose (4.16) to the reaction vessel;

Fill the electrodes EL_1 and EL_2 with the buffer + standard solution mixture;

Measure the potential difference (D_3) between the two electrodes;

Add 24 µL of enzyme solution (4.14) and fill electrode EL_2 with

- the buffer + standard solution + enzyme mixture;

After the time required for the enzymatic reaction, measure the potential difference (D_4) between the two electrodes;

Calculate the difference in pH, ΔpH_c for the calibration sample using the following formula:

$$\Delta pH_c = (D_4 - D_3) - \Delta pH_0$$

where

ΔpH_c = the difference in pH between two measurements D_3 and D_4 for the calibration sample minus the difference obtained for the blank;

D_3 = the value of the difference in pH between the two electrodes filled with the buffer/ reference solution mixture;

D_4 = the value of the difference in pH between the two electrodes, one of which is filled with the buffer/ reference solution and the other with the buffer/ reference solution /enzyme.

Calculate the slope of the calibration line:

$$s = C_u/\Delta pH_c$$

where

- C_u is the concentration of glucose in the standard solution expressed in g/L.

Check the validity of the calibration by analysing 10 µL of standard solution of glucose (4.16) in accordance with procedure (7.3). The result must range between ± 2% of the reference value. If not, repeat the calibration procedure.

7.3. Quantification

Fill the electrode compartments (EL_1 and EL_2) with the buffer solution (4.13)

Add 10 µL (high level) or 25 µL (mean level) (with the micropipette 5.9 or the preparer) of the sample solution to the reaction vessel;

Fill electrodes EL_1 and $EL_2$with the buffer + sample mixture;

Measure the potential difference (D_5) between the two electrodes;

Add 24 µL of the enzyme solution (4.14) and fill electrode EL_2 with the buffer mixture + sample + enzyme;

Measure the potential difference (D6) between the two electrodes;

Calculate the quantity of aqueous solution in the sample using the following formula:

$$w = s \times [(D_6 - D_5) - \Delta pH_0]$$

where

w = the quantity of aqueous solution in the sample (in g/L);

S is the slope determined by the calibration line;

ΔpH_o = the difference in pH between two measurements for the blank;

D_5 = the value of the difference in pH between the two electrodes filled with the sample/ reference solution;

D_6 = the value of the difference in pH between the two electrodes, one of which is filled with the buffer/sample and the other with the buffer/ sample /enzyme.

8. Expression of results

The results are expressed in g/L of glucose + fructose with one significant figure after the decimal point.

9. Precision

The details of the interlaboratory test on the precision of the method are summarized in appendix B.

9.1. Repeatability

The absolute difference between two individual results obtained in an identical matter tested by an operator using the same apparatus, in the shortest interval of time possible, shall not exceed the repeatability value *r* in 95% of the cases.

The value is: $r = 0.021x + 0.289$ where x is the content in g/L of glucose + fructose

9.2. Reproducibility

The absolute difference between two individual results obtained with an identical matter tested in two different laboratories, shall not exceed the reproducibility value of R in 95% of the cases.

The value is: R = 0.033x + 0.507 where w is the content in g/L of glucose + fructose

10. Other characteristics of the analysis

10.1. Detection and quantification limits

10.1.1. Detection limit

The detection limit is determined by using 10 series of three repetitions of an analytical blank and linear regression carried out with the wines of the precision test; it is equal to three standard deviations. In this case, the method gave as a result a detection limit of 0.03 g/L. Tests by successive dilutions confirmed this value.

10.1.2. Quantification limit

The quantification limit is determined by using 10 series of three repetitions of an analytical blank and linear regression carried out with the wines of the precision test; it is equal to ten standard deviations. In this case, the method gave as a result a quantification limit of 0.10 g/L. Tests by successive dilutions confirmed this value. The quantifications of white and red wine carried out by the laboratories that took part in the interlaboratory analysis also confirm these figures.

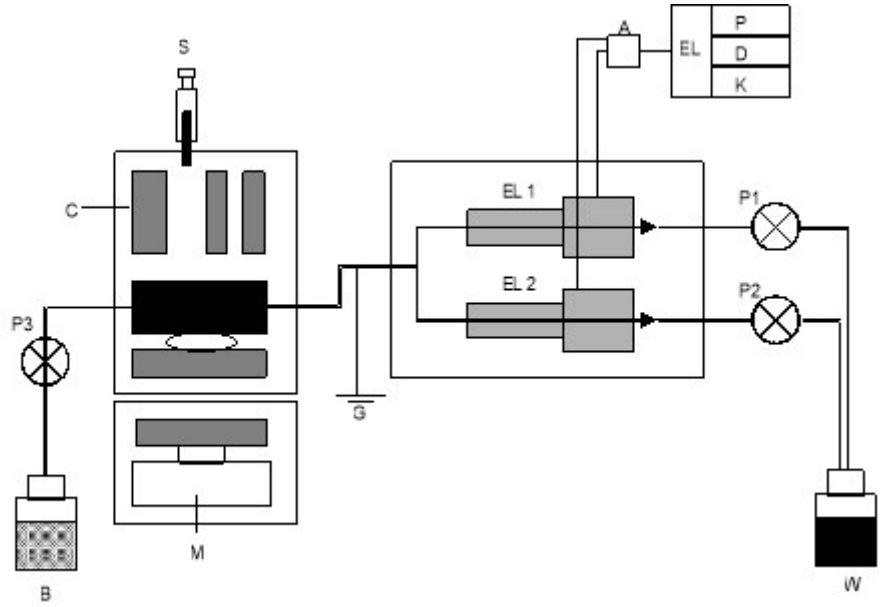
10.2. Accuracy

Accuracy is evaluated based on the average coverage rate calculated for the loaded wines analysed double-blind during the interlaboratory test (wines A, B, C, D, F and J). It is equal to 98.9% with a confidence interval of 0.22%.

11. Quality control

Quality controls can be carried out with certified reference materials, wines whose characteristics have been determined by consensus, or loaded wines regularly used in analytical series, and by following the related control charts.

Appendix A Diagram of the differential pH-metry apparatus



A: differential amplifier; B: buffer solution; C: mixing chamber; D: indicator; EL_1 and EL_2 capillary electrodes; EL: electronics; G: ground; K: keyboard; M: magnetic stirrer; P: printer; P_1 to P_3: peristaltic pumps; S: injection syringe for the sample and enzyme; W: waste.

Appendix B Statistical data obtained with the interlaboratory test results

In accordance with ISO 5725-2:1994, the following parameters were defined during an interlaboratory test. This test was carried out by the laboratory of the Inter-trade Committee for Champagne Wine in Epernay (France).

Year of the interlaboratory test: 2005

Number of laboratories: 13 double blind

Number of samples: 10

	Wine A	Wine B	Wine C	Wine D	Wine E	Wine F	Wine G	Wine H	Wine I	Wine J
Average in g/L	8.44	13.33	18.43	23.41	28.03	44.88	86.40	93.34	133.38	226.63
Number of laboratories	13	13	13	13	13	13	13	13	13	13

Number of laboratories after elimination of greatest dispersions	13	13	13	13	13	13	13	13	13	13
Standard deviation of repeatability	0.09	0.13	0.21	0.21	0.29	0.39	0.81	0.85	1.19	1.51
Repeatability limit	0.27	0.38	0.61	0.62	0.86	1.14	2.38	2.51	3.52	4.45
RSDr, 100%	1.08	0.97	1.13	0.91	1.04	0.86	0.94	0.91	0.89	0.67
HORRAT r	0.26	0.25	0.31	0.26	0.30	0.27	0.32	0.32	0.33	0.47
Standard deviation of reproducibility	0.17	0.27	0.37	0.59	0.55	0.45	1.27	1.43	1.74	2.69
Reproducibility limit	0.50	0.79	1.06	1.71	1.60	1.29	3.67	4.13	5.04	7.78
RSDR, 100%	2.05	2.05	1.99	2.54	1.97	1.00	1.47	1.53	1.31	1.19
HORRAT R	0.50	0.54	0.55	0.72	0.58	0.31	0.51	0.53	0.48	0.47

Types of samples:

Wine A: white wine naturally containing sugar, loaded with 2.50 g/L glucose and of 2.50 g/L of fructose;

Wine B: white wine naturally containing sugar (wine A), loaded with 5.00 g/L glucose and 50 g/L of fructose;

Wine C: white wine naturally containing sugar (wine A), loaded with 7.50 g/L glucose and 7,50 g/L of fructose;

Wine D: white wine naturally containing sugar (wine A), loaded with 10.0 g/L glucose and 10.0 g/L of fructose;

Wine E: aromatised wine;

Wine F: white wine naturally containing less than 0.4 g/L of sugar, loaded with 22.50 g/L glucose and 22.50 g/L of fructose;

Wine G: naturally sweet red wine;

Wine H: sweet white wine;

Wine I: basis wine;

Wine J: white wine naturally containing less than 0.4 g/L of sugar, loaded with 115.00 g/L glucose and 115.00 g/L of fructose;

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