RESOLUTION OENO 11/2006

WHOLE DETERMINATION OF GLUCOSE, FRUCTOSE AND SACCHAROSE CONTENT IN MUSTS AND WINES BY DIFFERENTIAL pH-METRY

The GENERAL ASSEMBLY

Considering Article 2 paragraph 2 iv of the Agreement of April 3, 2001 establishing the International Organization of Vine and Wine,

Upon the proposal of the Sub-commission of Methods of Analysis and Appraisal of Wine,

DECIDES to complete Annex A of the Compendium of International Methods of Analysis of Wine and Must with the following method and to adopt it in the form of a type IV method:

Title	Method
WHOLE DETERMINATION OF GLUCOSE, FRUCTOSE AND SACCHAROSE CONTENT IN WINES BY DIFFERENTIAL pH-METRY	**Type IV**

1. SCOPE

This method is applicable to the analysis of glucose and fructose in wines between 0 and 270 g/L.
This quantification is different from glucose and fructose quantification by its differential pH-metry which can not be substituted.

2. PRINCIPLE

The determination by differential pH-metry of glucose, fructose and saccharose content consists in the preliminary hydroloysis of saccharose by invertase, followed by 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

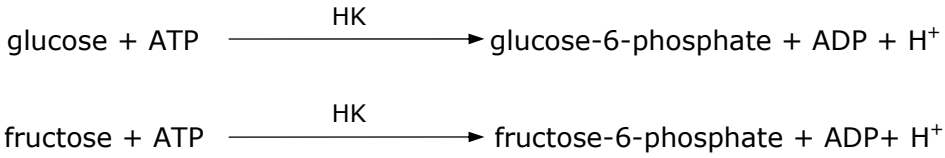
Possible traces of saccharose are hydrolysed by invertase (EC 3.2.1.26)

$$\text{Saccharose} \xrightarrow{\text{invertase}} \text{glucose + fructose}$$

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The glucose and fructose initially or consecutively present to invertase action are phosphorylated by adenosine triphosphate (ATP) during an enzymatic reaction catalysed by hexokinase (HK) (EC. 2.7.1.1)



4. REAGENTS

4.1 Demineralised Water (18 MΩ) 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 ($Na_3PO_4.12H_2O$) purity ≥ 99%

4.5 Sodium hydroxide (NaOH) purity ≥ 98%

4.6 Magnesium chloride with six water molecules ($MgCl_2.6H_2O$) 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 Invertase (EC 3.2.1.26) 1 mg ≅ 500 U (ex Sigma ref I-4504)

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

4.12 Glycerol purity ≥ 98%

4.13 Saccharose purity ≥ 99%

4.14 Reagent buffer pH 8.0 commercial (ex. DIFFCHAMB GEN 644) 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

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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.

4.15 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 300 mg ±1 mg (5.4) of invertase (4.10) 10 mg ± 1 mg (5.4) of hexokinase (4.11) in 3 mL of glycerol solution. Enzyme solution activity must be 50 000 U ± 100 U per ml for intervase and 480 U ± 50 U for hexokinase. The enzyme solution is stable for 6 months at 4°C.

4.16 PREPARATION OF REFERENCE SOLUTION

Place 17,100 g ± 0.01 g (5.4) of saccharose (4.13) (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 (5.4) of bronopol 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 171 g/L of saccharose. 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 foot

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 pipette, class A

5.8 Graduated 10-ml flask, class A

5.9 Automatic syringe pipettes, 25 and 50 µL

6. PREPARATION OF SAMPLES

Samples must not contain excessive suspended matter. If this occurs, the solution centrifuge and filter. Sparkling wines must be degassed

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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 (4.14) 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 (4.14); the potential difference between the two electrodes (D_1) must range between $\pm$ 150 mpH;
Add 32 µL of enzyme solution (4.15) 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_o for the blank using the following formula:

$\Delta pH_o = D_2 - D_1$
where
ΔpH_o = 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

Fill the electrode compartments (EL_1 and EL_2) with the buffer solution (4.14);
Add 10 µL (with the micropipette 5.9 or the preparer) of the standard saccharose solution (5) 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 32 µL of enzyme solution (4.15) 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:

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

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 saccharose in the standard solution expressed in g/L.

Check the validity of the calibration by analysing 10 µL of standard solution (ML) of saccharose (5) according to the procedure (8.3). 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 (4.14)
Add 10 µL (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 32 µL of the enzyme solution (4.15) 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:

$w = s \times [(D_6 - D_5) - \Delta pH_o]$
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 with one significant figure after the decimal point.

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9 CHARACTERISTICS OF THE ANALYSIS

Due to the hydrolysis of saccharose in wines and musts, it is not possible to organise an inter-laboratory analysis according to the OIV protocol.
Inter-laboratory studies of this method demonstrate that for saccharose, the linearity between 0 and 250 g/l, a detection limit of 0.2 g/l, a quantification limit of 0.6 g/l, repeatability of 0.0837x -0.0249 g/l and reproducibility of 0.0935x -0.073 g/l (saccharose content).

10 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.

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Appendix A

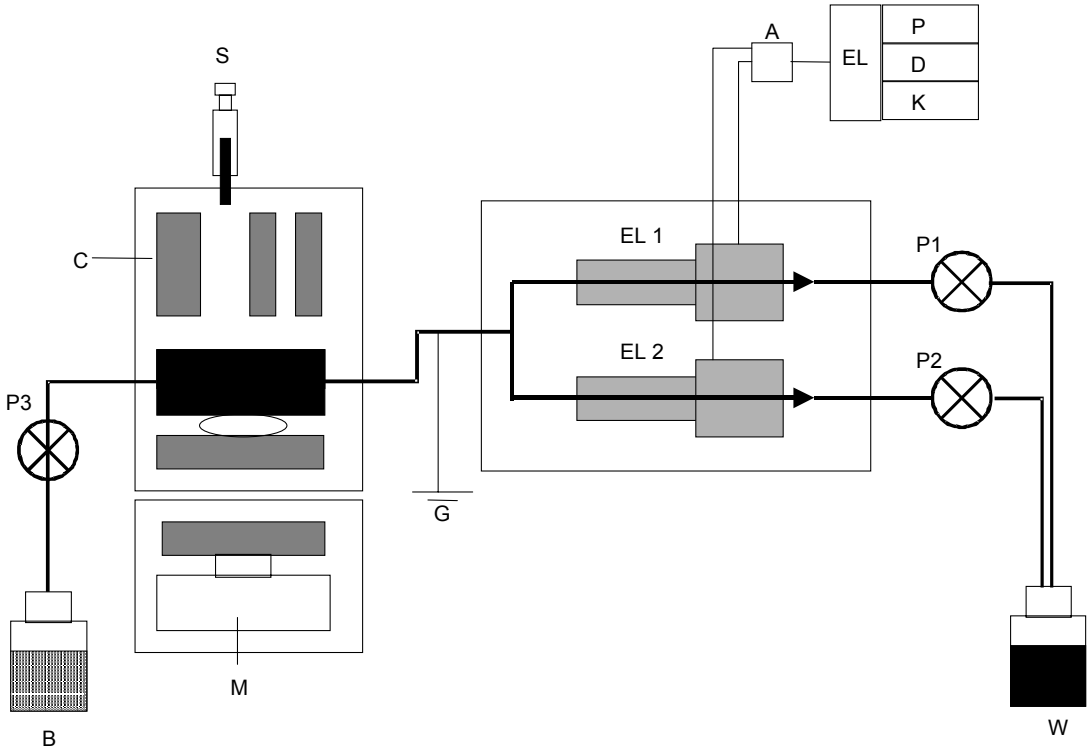


Diagram of the differential pH-metry apparatus

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.

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Appendix B

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