RESOLUTION OENO 5/2007

CODEX - GLYCOSIDASE

The GENERAL ASSEMBLY

IN VIEW of article 2, paragraph 2 IV of the Agreement of 3 April 2001, by which the International Organisation of Vine and Wine was founded,

Following a proposal made by the Sub-Commission of Methods of Analysis and Appraisal of Wine and the Specification of Oenological Products expert group,

HAS HEREBY DECIDED to add the following monograph to the international Oenological Codex:

GLYCOSIDASE

(activity β-D-glucosidase)

(EC 3.2.1.21 – CAS no. 9001-22-3)

General specifications

These enzymes are not found in a pure state but they are present within an enzymatic complex. Unless otherwise stipulated, the specifications must comply with Oeno resolution 14/2003 concerning the general specifications for enzymatic preparations included in the international oenological Codex.

1. Origin and oenological application

Enzymes of the glycosidase type are used to reveal the flavours of musts and wines based on their glycosylated precursors.

The enzymatic preparations containing these activities come from directed fermentations of Aspergillus Niger.

Secondary activities: proteases, cinnamoyl esterase (the latter must be as limited as possible) the measurement method of the cinnamoyl esterase activity is described elsewhere, in this case, the 50% clause can be applied. (Resolution Oeno 14/2003 4.1)

2. Scope

The determination method was developed using a commercially available β-D-

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glucosidase (5.5). The conditions and the method were developed for application to the commercial enzymatic preparations such as those found on the oenological market.

3. Principle

The enzymatic hydrolysis of p-nitrophenyl-¤-D-Glucopyranoside, which is colourless, releases glucose and para-Nitrophenol (p-Np); the latter turns yellow in the presence of sodium carbonate, the absorbance of which is measured at 400 nm.

4. Apparatus

4.1. magnetic stirrer

4.2. water bath at 30°C

4.3. water bath at 100°C

4.4. cuvets with a 1-cm optical path length, for single use, for spectrophotometer, for measurement in the visible spectrum

4.5. crushed ice

4.6. precision syringe 500 – 5000 µ

4.7. precision syringe 100 µl

4.8. precision syringe 1000 µl

4.9. spectrophotometer

4.10. Eppendorf tubes

4.11. 100-mL graduated flask

4.12. pH-meter

4.13. cold room at 4°C

4.14. metal rack for Eppendorf tubes

4.15. carded cotton

4.16. Kraft paper

4.17. agitator of the vortex

4.18. chronometer

4.19. 15-mL glass tubes

5. Products

5.1. Sodium carbonate (Na_2CO_3 99.5% pure - PM:105.99 g/mole)

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5.2. Sodium acetate (CH_3 COONa 99% pure - PM: 82g/mole)

5.3. Acetic acid (CH_3 COOH 96% pure - PM: 60g/mole)

5.4. p-nitrophenyl-β-D-Glucopyranoside (Fluka, ref. 73676) as an example

5.5. β-D-glucosidase (Fluka; 250 mg; 6.3 U/mg, ref. 49290) as an example. One unit corresponds to the quantity of enzyme required to release 1 µmole of glucose per minute with pH 5 and 35°C.

5.6. p-nitrophenol (p - Np) ($C_6H_5NO_3$ 99.5% pure - PM: 139.11 g/mole)

5.7. Distilled water

5.8. Commercial enzymatic preparation for analysis

6. Solutions

6.1. Sodium acetate buffer (100 mM, pH 4.2)

It consists of solutions A and B.

6.1.1. Solution A: introduce 0.5 g of sodium acetate (5.2) into 60 ml of distilled water (5.7)

6.1.2. Solution B: introduce 1 ml of acetic acid (5.3) into 175 mL of distilled water (5.7)

6.1.3 Preparation of the sodium acetate buffer: mix 47.8 ml of solution A (6.1.1) + 152 ml of solution B (6.1.2).

Check the pH of the buffer using a pH-meter (4.12).

Maintain at 4°C

6.2. Solution of p-nitrophenyl-□-D-Glucopyranoside 4mM

Place 0.096 g of p-nitrophenyl-□-D-Glucopyranoside (5.4) in 80 mL of sodium acetate buffer (6.1.).

6.3. Sodium carbonate solution 1M

Dissolve 10.6 g of sodium carbonate (5.1) in 100 mL of water distilled (5.7) in a 100-ml graduated flask (4.11). The solution can be maintained at 4°C (4.13).

6.4. Stock solution of p-nitrophenol (p-Np) at 125 µg/ml Dissolve 0.01 g of p-Np (5.6) in 80 mL of distilled water (5.7). The stock solution must be prepared extemporaneously.

7. Preparation of the standard range of p-nitrophenol (p - Np) from 0 to 50 µg/ml

It is made up using the stock solution of p-nitrophenol (p - Np) (6.4.) as indicated in table 1.

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Table 1: Standard range of para-Nitrophenol

Quantity of p-Np (µg)	0	2	4	6	8	10
P-Np concentration (µg/mL)	0	10	20	30	40	50
P-Np concentration (µmol/mL)	0	.07222	0.14	0.22	0.29	0.36
Volume of stock solution (6.4) (µl)	0	16	32	48	64	80
Distilled water (5.7) (µl)	200	184	168	152	136	120

8. Preparation of the sample

It is important to homogenise the enzymatic preparation before sampling, by upturning the container for example. The enzymatic solution and the blanks will have to be prepared extemporaneously.

8.1. Enzymatic solution with 10 g/l

Place 1 g of commercial preparation (5.8) in a 100-mL graduated flask (4.11), make up with distilled water (5.7), and stir (4.1) in order to obtain a homogeneous mixture.

8.2. Blank denatured by heating

Place 10 mL of the enzymatic solution at 2 g/l (8.1) in a 15 mL tube (4.19), plug with carded cotton (4.15) covered with Kraft paper (4.16) and immerse the tube for 5 minutes in the water bath to 100°C (4.3).

9. Procedure

9.1. Enzymatic reaction: The tubes are produced at least in duplicate..

In 5 Eppendorf tubes (4.10) numbered 1 to 5, placed in a rack (4.14) in ice crushed (4.5) introduce

100 µl of the solution of p-nitrophenyl-□-D-Glucopyranoside (6.2), using a precision syringe (4.7),

100 µl of the enzymatic solution with 2 g/l (8.1), start the chronometer (4.18)

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After stirring (4.17), the Eppendorf tubes are placed in the water bath at 30°C (4.2)

- for 1 min. for tube no. 1
- for 2 min. for tube no.2
- for 5 min. for tube no.3
- for 10 min. for tube no.4
- for 15 min. for tube no.5

The reaction is stopped by placing each of the tubes numbered from 1 to 5 immediately after they have been removed from the water bath at 30°C, in a bath of crushed ice (4.5)

9.2. Determination of p-nitrophenol released

From the Eppendorf tubes containing the various reactional mediums (9.1)
Add 600 µl of sodium carbonate solution (6.3), using a precision syringe (4.8),
1.7 ml of distilled water (5.7), using a precision syringe (4.6),
Place the resulting mixture in a tank (4.4).
Immediately measure the absorbance at 400 nm, using a spectrophotometer (4.9)

9.3. Blanks

Proceed as described in 9.1 by replacing the enzymatic solution with 2 g/l (8.1) by the blank denatured by heat (8.2). The ideal situation is to carry out the enzymatic reaction of the blank at the same time as that of the enzymatic solution.

9.4. Standard range

Proceed as described in 9.2 by replacing the reactional medium (9.1) by the various mediums of the standard range of p-nitrophenol from 0 to 50 µg/mL (7).

10. Calculations

10.1. Determining the kinetics

In general, calculating the enzymatic activity can only be done when the substrate and the enzyme are not in limiting quantities. This therefore refers to the ascending phase of the kinetic representation: the enzymatic activity is linear in time. Otherwise, the

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activity would be underestimated (Figure 1).

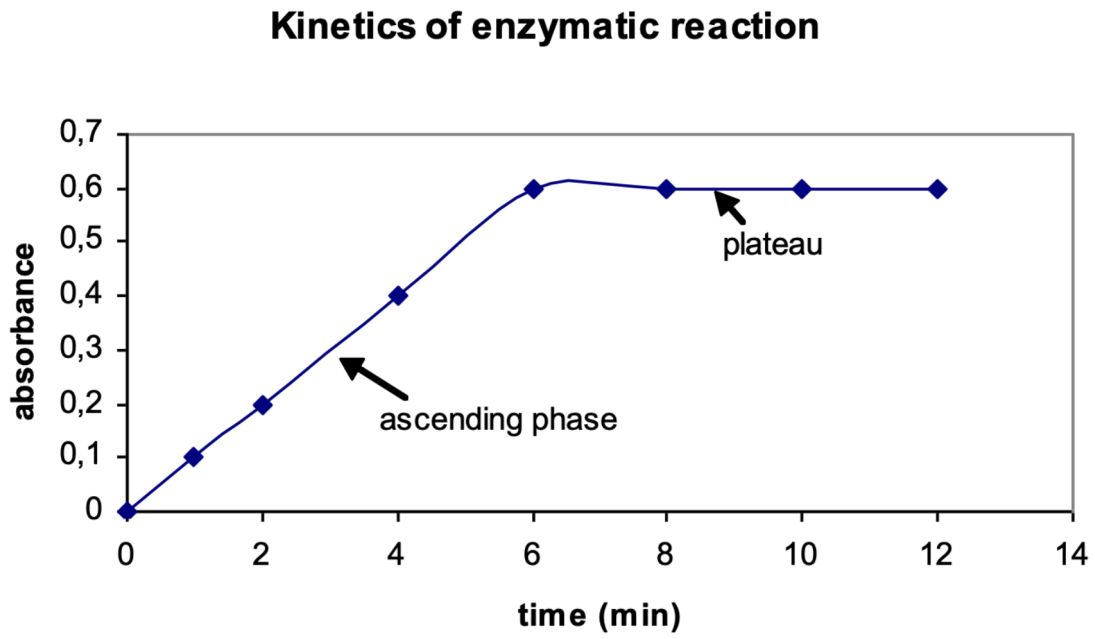

Figure 1: Kinetics of an enzymatic reaction

The kinetics are determined over 12 minutes. The activity concerned is measured at T=1 min T=2 min, T=4 min, T=6 min T=8 min T=10 min, T=12 min.

After determining the kinetics of the enzymatic reaction, plot the curve for the variation in absorbance in relation to reaction time. The absorbance corresponds to the difference between the absorbance at time T of the enzymatic preparation and that of the corresponding blank. Then calculate the equation (1) of the straight regression line, taking into account only the points of the ascending phase (see figure 1).

10.2. Producing the calibration line

The calibration line corresponds to plotting a graph whose X-coordinates are the various concentrations of the standard range of p.nitrophenol (from 0 to 0.36 µmole/ml) and whose Y-coordinates are the corresponding values of optical densities, obtained in 9.4. Then calculate the Q/T slope of the straight regression line (2) resulting from the linearity of the data of the graph.

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10.3. Calculating the enzymatic activity

Based on the straight regression line (1) calculate the absorbance for an average time T (for example 4 min. in the case of figure 1) deduct from it the quantity Q of p.nitrophenol released (in µmoles) for this intermediate time using equation (2).

The formula used to calculate the enzymatic activity in U/g of the preparation is as follows

- Activity in U/g = $1000 \times (Q/T)/(V \times C)$

Where Q: quantity of p. nitrophenol formed in µmoles during time T (min)

V: quantity of enzymatic solution introduced (ml) here 0.1 ml

C: concentration of the enzymatic solution (g/l) here 2 g/l

It is then possible to express the enzymatic activity in nanokatals. This unit corresponds to the number of nanomoles of product formed per second under the conditions defined by the determination protocols and therefore:

- Activity in nkat/g = (activity in U/g) * (1000/60)

11. Characteristics

The repeatability of the method is estimated using the mean standard deviation of the absorbance values resulting from the same sampling of the enzymatic preparation, proportioned 5 times. In this way, to proportion □-D-glucosidase the mean standard deviation of the values is 0.01 with a percentage error of 8.43, in which the % error corresponds to:

$$\frac{(mean\ standard\ deviation\ of\ values\ \times 100)}{mean\ test\ value}$$

In this way, the determination method as presented is considered repeatable.

The reproducibility tests were carried out using 2 enzymatic preparations with 5 samplings for each.

2 tests were used in order to determine the satisfactory reproducibility of the method:

- variance analysis (the study of the probability of the occurrence of differences

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between samplings). Variance analysis is a statistical method used to test the homogeneity hypothesis of a series of K averages. Performing the variance analysis consists in determining if the "treatment" effect is "significant or not"

- the power of the test for the first species of risk α (5%) - first species of risk □ is the risk of deciding that identical treatments are in fact different.

If the power is low (≅ 20%), this means that no difference has been detected between treatments, but there is little chance of seeing a difference if one did in fact exist.

If the power is high (≅ 80%), this means that no difference has been detected between the treatments, but, if there was one, we have the means of seeing it.

The results are given in table 2.

Determination	Variance analysis hypotheses	Probability	Power of test (□= 5%)	Newman-Keuls test (*)	Bonferroni test (**)
□-D-glucosidase	Adhered to	0.0285	42%	Non Significant	Non Significant

Table 2: Variance analysis – study of the sampling effect

* Newmann-Keuls test: this comparison test of means is used to constitute homogeneous groups of treatments: those belonging to the same group are regarded as not being different to risk α of the first species selected

** Bonferroni test: also referred to as the "corrected T test", the Bonferroni test is used to carry out all the comparisons of pairs of means, i.e., (t (t-1))/2 comparisons before treatments, respecting the risk α of the first species selected.

In this way, the tests set up are used to see a difference if there really is one (high power test); in addition, the determination method involves a probability of occurrence of a discrepancy in activity (between samplings) lower than 5%, reinforced by belonging to the same group (Newmann-Keuls test not significant) and considered not to be different to the first species of risk α (Bonferroni test not significant).

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