RESOLUTION OIV-OENO 451-2012

DETERMINATION OF GLYCOSIDASE ACTIVITIES IN ENZYMATIC PREPARATIONS - REVISION OF THE MONOGRAPH (OENO 5/2007)

THE GENERAL ASSEMBLY,

IN VIEW of article 2, paragraph 2 iv of the agreement dated 3rd of April, 2001, by which the International Organization of Vine and Wine was founded,

CONSIDERING resolution OENO 5/2007 concerning the determination of glycosidase activity in enzymatic preparations

Upon proposal by the "Specification of oenological products" expert group,

CONSIDERING the fact that aromatic molecules are partially in the form of glycosides; that they are for the main part associated with glucose; that the measurement of enzymatic activity sufficient to break this specific bond has been described in the monograph (Oeno

5/2007). However, this activity is not really functional if the glucose is itself bound to another type of sugar (which is the case for most aromatic precursors). These are essentially apiose, arabinose, rhamnose and xylose.

In order to measure the true efficiency of an enzymatic preparation so as to obtain the aromatic potential of the grape or wine, the monograph concerning ß-D-glucosidase activity should be replaced by a new one describing glycosidases active on heterosidic aroma precursors.

DECIDES to include in the monograph relating to the determination of glycosidase activity in enzymatic preparations contained in the International Oenological Codex the following appendix concerning the measurement of arabinofuranosidase, ß-D-galactosidase, rhamnosidase and xylosidase activities.

Determination of various glycosidase activities in enzyme preparations

β-D-galactosidase (EC 3.2.1.23 – CAS n° 9031-11-2)

α -L-arabinofuranosidase (EC 3.2.1.55 – CAS n° 9067-74-7)

α -L-rhamnosidase (EC 3.2.1.40 – CAS n° 37288-35-0)

β -D-xylosidase (EC 3.2.1.34 – CAS n° 9025-53-0)

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General specifications

These enzymatic activities are usually present among other activities within an enzymatic complex. Unless otherwise stipulated, the specifications must comply with the resolution Oeno 365 - 2009 concerning the general specifications for enzymatic preparations included in the International Oenological Codex.

1. Origin

Reference is made to paragraph 5 “Source of enzyme and fermentation environment” of the general monograph on Enzymatic preparation

The enzymatic preparations containing these activities are produced by directed fermentations of Aspergillus niger for example.

2. Scope/ Applications

Reference is made to the International Code of Oenological Practices, Oeno 16/04 and 17/04.

The glycosidase activities are used to reveal and enhance the flavours of wines based on hydrolysis of the sugar part of their glycosylated precursors. The enzymes can also be added to the must but their technological efficiencies will become active only after completion of the alcoholic fermentation.

3. Principle

Available enzymatic preparations with glycosidase activity contain enzymes that are able to hydrolyse the glycosidic bonds between glucose and other types of sugar, and in particular: apiose, galactose, arabinose, rhamnose and xylose- which then liberate the aromatic compounds contained in glucose by means of glycosidase activity. Similarly, the enzymes are capable of hydrolysing the bond of synthetic compounds that includes these various types of osidic compounds and p-nitrophenol. This enables to measure these different activities.

Determination of β-D-galactosidase activity

The enzymatic hydrolysis of β-D- galactopyranoside of p-nitrophenyl, which is colourless, liberates galactose and para-nitrophenol (p-Np); the latter takes on a yellow colour when mixed with sodium carbonate, the absorbance of which is measured at 400nm.

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Determination of α-L-arabinofuranosidase activity

The enzymatic hydrolysis of α-L-arabinofuranoside of p-nitrophenyl, which is colourless, liberates arabinose and p-nitrophenol (p-Np); the latter takes on a yellow colour when mixed with sodium carbonate, the absorbance of which is measured at 400nm.

Determination of α -L-rhamnosidase activity

The enzymatic hydrolysis of α-L-rhamnopyranoside of p-nitrophenyl, which is colourless, liberates rhamnose and p-nitrophenol (p-Np); the latter takes on a yellow colour when mixed with sodium carbonate, the absorbance of which is measured at 400nm.

Determination of β -D-xylosidase activity

The enzymatic hydrolysis of β-D-xylopyranoside of p-nitrophenyl, which is colourless, liberates xylose and p-nitrophenol (p-Np); the latter takes on a yellow colour when mixed with sodium carbonate, the absorbance of which is measured at 400nm.

4. Apparatus

4.1. magnetic stirrer

4.2. 40°C water bath

4.3. 100°C water bath

4.4. single-use 1 cm optical path vats for spectrophotometer measurement in the visible range

4.5. crushed ice

4.6. precision syringes 500 – 5000 µl

4.7. precision syringe 100 µl

4.8. precision syringe 1000 µl

4.9. spectrophotometer

4.10. eppendorf tube

4.11. 100 ml volumetric flask

4.12. pH meter

4.13. 4°C cold room

4.14. metal tray for eppendorf tubes

4.15. absorbent cotton

4.16. Kraft paper

4.17. vortex type stirrer

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4.18. timer

4.19. 15 ml glass tubes

5. Products

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

5.2. Sodium acetate (pure $NaCH_3COO$ at 99% - PM: 82g/mole)

5.3. Acetic acid (pure CH_3COOH at 96% - PM: 60g/mole)

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

5.5. Distilled water

5.6. Commercial enzymatic preparation to be analysed, and depending on the measurement of the considered activity:

5.7a. β -D-galactopyranoside de p-nitrophenyl (Sigma ref. N1252, 250 mg) as an example

5.7b. α-L-arabinofuranoside de p-nitrophenyl (Sigma ref. N3641, 10 mg) as an example

5.7c. α -L-rhamnopyranoside de p-nitrophenyl (Sigma ref. N7763, 100 mg) as an example

5.7d. β -D-xylopyranoside de p-nitrophenyl (Sigma ref. N2132, 500 mg) as an example

6. Solutions

For the determination of α -L-arabinofuranosidase or α-L- rhamnosidase

6.1. Sodium acetate buffer (100 mM, pH 4.4) It is made of solutions A and B.

6.1.1. Solution A: add 0.984 g of sodium acetate (5.2) in 60 ml of distilled water (5.6)

6.1.2. Solution B: add 2 ml of acetic acid (5.3) in 175 ml of distilled water (5.6)

6.1.3. Preparation of the sodium acetate buffer: Add 78 ml of solution A (6.1.1) + 122 ml of solution B (6.1.2).

Control the pH of the buffer with the pH meter (4.12).

Keep at 4°C

For the determination of □-D-galactosidase or □-D-xylosidase activity

6.1. Sodium acetate buffer (100 mM, pH 4.0) It is made of solutions A and B.

6.1.1. Solution A: add 0.984 g of sodium acetate (5.2) in 60 ml of distilled water (5.6)

6.1.2. Solution B: add 2 ml of acetic acid (5.3) in 175 ml of distilled water (5.6)

6.1.3. Preparation of the sodium acetate buffer: Add 36 ml of solution A (6.1.1) + 164 ml of solution B (6.1.2).

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Control the pH of the buffer with the pH meter (4.12).

Keep at 4°C

6.2. Reagent solution (depending on the measurement of the considered enzymatic activity)

a) Solution of p-nitrophenyl α-L-arabinofuranoside 4 mM

Add 0.086 g of p-nitrophenyl α-L-arabinofuranoside (5.4) in 80 ml of sodium acetate buffer (6.1.).

b) Solution of p-n i t rop h en yl □-D-galactopyranoside 4 mM

Add 0.096 g of p-nitrophenyl □-D-galactopyranoside (5.4) in 80 ml of sodium acetate buffer (6.1).

c) Solution of p-nitrophenyl α-L-rhamnopyranoside 4 mM

Add 0.091 g of p-nitrophenyl α-L-rhamnopyranoside (5.4) in 80 ml of sodium acetate buffer (6.1.).

d) Solution of p-n i t rop hen yl □-D-xylopyranoside 4 mM

Add 0.0868 g of p-nitrophenyl □-D-xylopyranoside (5.4) in 80 ml of sodium acetate buffer (6.1).

6.3. Solution of sodium carbonate 1M

Dissolve 10.6 g of sodium carbonate (5.1) in 100 ml of distilled water (5.6) in a 100 ml volumetric flask (4.11). The solution may be kept at 4°C (4.13).

6.4. Stock solution of p-nitrophenol at 125 □g/ml

Dissolve 0.01 g of p-nitrophenol (5.5) in 80 ml of distilled water (5.6). The stock solution must be prepared extemporaneously.

7. Preparation of the standard range of p-nitrophenol from 0 to 100 µg/ml

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

Table 1: Standard range of p-nitrophenol (p.Np)

Quantity of p-Np (µg)	0	4	8	12	16	20
Concentration of p-Np (µg/ml)	0	20	40	60	80	100

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Concentration of p-Np (µmol/ml)	0	0.14	0.2	0.43	0.5	0.72
			9		8	
Volume of stock solution (6.4) (µl)	0	16	32	48	64	80
Distilled water (5.5) (µl)	200	184	168	152	136	120

8. Preparation of the sample

It is important that the enzymatic preparation be homogeneous before sampling, by shaking it for example. The enzymatic solution and whites are to be prepared extemporaneously.

8.1. Enzymatic solutions

For the determination of α-L-rhamnosidase or β-D- xylosidase activity

10 g/l enzymatic solution

Put 1 g of commercially available preparation (5.6) in a 100 ml volumetric flask (4.11), add distilled water (5.5), and stir (4.1) in order to achieve a homogeneous solution.

For the determination of α-L-arabinofuranosidase activity

1 g/l enzymatic solution

Put 100 mg of commercially available preparation (5.6) in a 100 ml volumetric flask (4.11), add distilled water (5.5), and stir (4.1) in order to achieve a homogeneous solution.

For the determination of β-D-galactosidase activity

2 g/l enzymatic solution

Put 100 mg of commercially available preparation (5.6) in a 100 ml volumetric flask (4.11), add distilled water (5.5), and stir (4.1) in order to achieve a homogeneous solution.

8.2. Denatured white through heating

Put 10 ml of the enzymatic solution (8.1) in a 15 ml tube (4.19), plug with absorbent cotton (4.15) covered with Kraft paper (4.16) and immerse the tube for 5 minutes in the

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100°C water bath (4.3).

9. Procedure

9.1. Enzymatic reaction: The tubes must be at least doubled.

In 6 eppendorf tubes (4.10) numbered from 1 to 6 and placed in a tray (4.14) of crushed ice (4.5), introduce

- 100 µl of the considered reagent solution (6.2), with a precision syringe (4.7),
- 100 µl of the corresponding enzymatic solution (8.1), start the timer (4.18)

After stirring (4.17), the eppendorf tubes are placed in the 40°C water bath (4.2)

- for 2 mn in tube n° 1 for 5 mn in tube n° 2
- for 10 mn in tube n° 3 for 15 mn in tube n° 4 for 20 mn in tube n° 5 for 30 mn in tube n° 6

The reaction is stopped by placing each numbered (1-6) tube immediately after extraction from the 40°C water bath in the tray of crushed ice (4.5).

9.2. Determination of liberated p-nitrophenol

With the eppendorf tubes containing the various reactive media (9.1)

- add 600 µl of the considered reagent solution (6.3), with a precision syringe (4.8), and
- 1.7 ml of distilled water (5.5) with a precision syringe (4.6), Place the resulting mixture in a vat (4.4).

Immediately measure the absorbance at 400 nm with a spectrophotometer (4.9)

(This can also be simplified by indicating: See point 8.2 pertaining to the measurement of β-D-glycosidase activity)

9.3. blank

Proceed as per indications given in point 9.1 by replacing the enzymatic solution (8.1) with whites denatured by heating (8.2). Ideally, the enzymatic reaction of whites should be carried out at the same time as the reaction of the enzymatic solution.

9.4. Standard range

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Proceed as described for point 9.2 by replacing the reactive medium (9.1) with various media of the standard range of p-nitrophenol from 0 to 100 µg/ml (7).

10. Calculations

10.1. Chemical kinetics

Generally, the calculation of the enzymatic activity can only be carried out when the substrate and the enzyme are not in limiting quantities. This corresponds to the ascending phase of the kinetic representation: the enzymatic activity is linear in time. If this were not to be the case, the activity would be underestimated (Illustration 1).

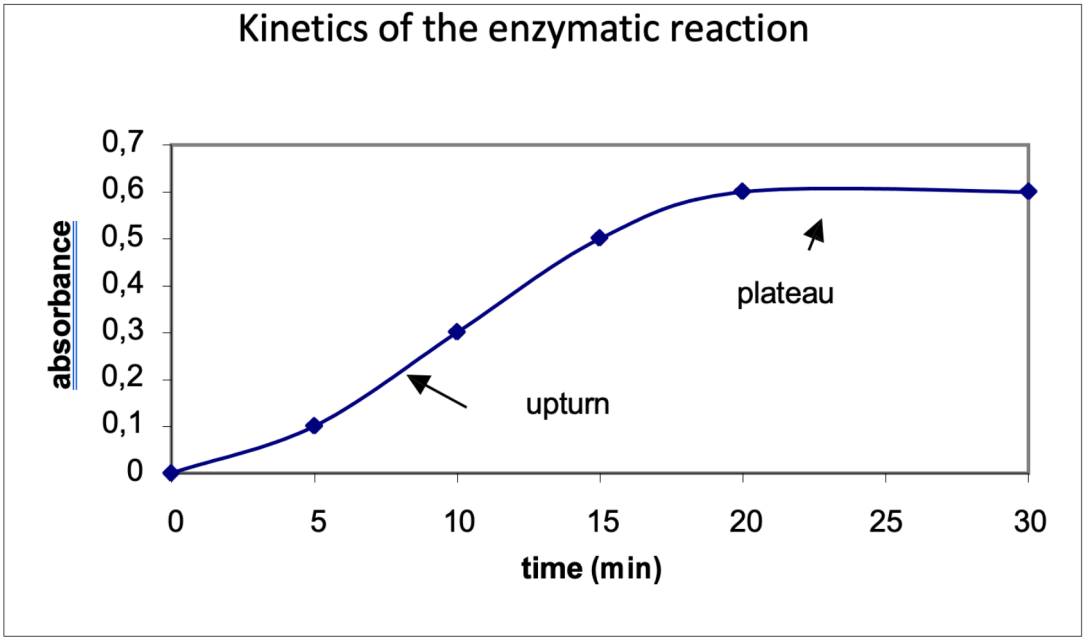

Illustration 1: Kinetic rate of an enzymatic rate

A kinetic calculation is performed for 30 minutes. The activity under consideration is measured at T=2 min, T=5 min, T=10 min, T=15 min, T=20 min, T=30 min.

After having calculated the kinetic rate of the enzymatic reaction, establish the variation curve of absorbance according to reaction times. Absorbance is the difference between absorbance at time T of the enzymatic preparation and the corresponding white.

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Then calculate the equation (1) of the regression curve by considering only the points of the ascending phase (see illustration 1).

10.2. Establishing the standard line

The standard calibration line is established in a graph where the x-axis represents the various concentrations of the standard range of the p-nitrophenol (0 to 0.72 µmole/ml) and the y-axis represents the various corresponding optical densities established in 8.4. Then calculate the regression curve (2) that results from the linearity of the graph's data.

10.3. Calculation of enzymatic activities

Based on the regression curve (1), calculate the absorbance for an average time of T (for example 4 mn in the case of illustration 1) and deduce the Q quantity of liberated p- nitrophenol (in µmoles) for this intermediate time with equation (2).

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

- Activity at U/g = Q/T/V/C*1000

Where

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

V: quantity of introduced enzymatic solution (ml), in this instance 0.1 ml

C: concentration of the enzymatic solution (g/l), in this instance 10 g/l

It then becomes possible to represent the enzymatic activity in nanokatal. This unit corresponds to the number of nanomoles of the amount of product created per second in the conditions defined in determination protocols, and therefore:

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

11. Reproducibility

The reproducibility of the method is estimated with the average of standard deviations of absorbance values resulting from a sample taken from the same enzymatic preparation, determined five times.

The table below summarises the results:

Activity	average of values' standard deviations	error percentage (%)
□-L-arabinofuranosidase	0	5
□-D-galactosidase	0.03	3.78
□-L-rhamnosidase	0.001	4.66
□-D-xylosidase	0.03	3.78

The % of error corresponds to:

$$\frac{(average\ of\ standard\ deviations\ of\ the\ values\ \times 100}{average\ of\ trial\ values)}$$

Hence, the determination method as presented herein is deemed to be reproducible.

The reproducibility trails were carried out with 2 enzymatic preparations and 5 samplings for each.

Two tests were used to determine the proper reproducibility of the method:

- the analysis of variance (the study of the probability of deviations between samples). The variance analysis is a statistical method that enables to test the homogeneity hypothesis of a set of average k values. The variance analysis consists in determining whether the "treatment" effect is "significant or not"
- the strength of the trial with type I error (5%) – type I error is the risk of deciding that identical treatments are different
- If the strength is feeble (□ 20%), this means that no difference has been detected between treatments, but there is little chance of seeing a difference if there actually were one.

If the strength is high (□ 80%), this means that no difference has been detected

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between treatments, but we would have the means of seeing it if such a difference were present.

The results are given in table 2.

Determinations	Hypotheses of variance analysis	Probability	Strength of the trial (α = 5%)	Newman-Keuls test (*)	Bonferroni test (**)
□-L-arabinofuranosidase	Satisfied	0.0125	45%	Not significant	Not significant
□-D-galactosidase	Satisfied	0.01	75%	Not significant	Not significant
□-L-rhamnopyranoside	Satisfied	0.006	65%	Not significant	Not significant
□-D-xylosidase	Satisfied	0.0253	73%	Not significant	Not significant

Table 2: Variance analysis – stuffy of the sampling effect

* Newman-Keuls test: this test is used to compare averages and enables to establish homogeneous treatment groups: those that belong to a same group are considered as not different to the chosen type I error

** Bonferroni test: also known as the “Bonferroni correction” the Bonferroni test enables to carry out all 2 on 2 average comparisons. i.e. (t(t-1))/2 comparisons before treatments. respecting the chosen type I error.

Therefore. the tests conducted enable to identify a difference if such a difference exists (high trial strength); furthermore the determination method presents the probability of activity deviations (from one sampling to the next) of less than 5% reinforced by belonging to the same group (non-significant Newmann-Keuls test) and considered to be not different from type I error (non-significant Bonferroni test).

12. Bibliography

1. M. Lecas. Thèse de Doctorat. 1994. Composition et structure des polymères constitutifs des parois cellulaires de la pellicule de la baie de raisin. Application d’enzymes fongiques liquéfiant les parois à fins d’extraction des substances

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2. I. Dugelay. Thèse de Doctorat. 1993. L'arôme du raisin : étude des précurseurs hétérosidiques et des activités enzymatiques exogènes impliquées dans leur hydrolyse – Applications technologiques.

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