RESOLUTION OENO 9/2008

CODEX - PECTIN METHYL ESTERASE

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 Specifications of Oenological Procedures expert group,

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

PECTIN METHYL ESTERASE

(Pectin Methyl-Esterase Activity) (PME)

(EC. 3.1.1.11 – CAS N° 9025-98-3)

General specifications

These enzymes are not found in a pure state but they are present within an enzyme 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 scope

These enzyme activities are used to support grape maceration, to clarify musts and wines, to improve the filterability of musts and wines, and grape pressing.

The enzyme preparations containing such activity come from directed fermentations of Aspergillus niger.

Principle enzyme activities accompanying the pectin methyl esterase activity include:

- Polygalacturonase
- Pectin lyase

Secondary enzyme activities: Various enzyme activities such as arabanases, galactanases, rhamnosidasesand xylanases, as well as cellulases, can be considered as

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secondary enzyme activities, but are also extremely useful for the hydrolysis of pectic substances. In this case, given their usefulness, it is not appropriate to apply the clause of resolution 14/2003 requiring that the sum of secondary enzyme activities should not be higher than 50% of the sum of the enzyme activities needed by the designated function, since they usefully contribute to reaching that objective.

On the contrary, the 50% clause is applicable for the following secondary enzyme activities: proteases, beta-glucosidases

2. Scope

The method of determination was developed using a commercially available pectin esterase (5.2). The conditions and the method were developed for application to the commercial enzyme preparations such as those found on the oenological market.

3. Principle

The enzyme activity of demethylation of the pectin results in the appearance of free carboxyl groups associated with the galacturonic acids making up the chains.

The pectin methyl-esterase activity is estimated by determination of the methanol according to the Klavons & Bennet method (1986). The alcohol oxydase of Pichia pastoris is specific to primary alcohols with a low molecular weight and catalyses the oxidation of the methanol into formaldehyde. 2,4-Pentanedione condenses exclusively with aldehydes of low molecular weight such as formaldehyde, forming a chromophore absorbing at 412 nm.

4. Equipment

4.1. water bath at 25°C

4.2. water bath at 30°C

4.3. water bath at 60°C

4.4. water bath at 100°C

4.5. 100-ml cylindrical flask

4.6. stop-watch

4.7. disposable spectrophotometer cuvettes with a 1-cm optical path length, for measurement in the visible spectrum

4.8. 1-L graduated flask

4.9. 100-ml graduated flask

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4.10. pH-meter
4.11. 500-5000 µl precision syringe
4.12. 100-1000 µl precision syringe
4.13. 0-200 µl precision syringe
4.14. 0-20 µl precision syringe
4.15. spectrophotometer
4.16. 15-ml sealed glass screw-top test tubes
4.17. metal rack for 15 ml test tubes
4.18. Vortex-type mixer
4.19. magnetic stirrer

5. Reagents

5.1. citrus fruit pectin with a degree of esterification of 63-66%. (Pectins ex-citrus: Fluka, ref: 76280 as an example).
5.2. orange peel pectin esterase (Fluka; 20 U/mg, ref: 76286 as an example).
5.3. sodium acetate (CH_3COONa 99% pure - MW = 82g/mole)
5.4. acetic acid (CH_3COOH 96% pure - MW = 60 g/mole, density = 1.058)
5.5. alcohol oxydase of Pichia Pastoris (Sigma, 250 U; 0.2 ml, ref: A2404 as an example). One unit of alcohol oxydase oxidizes one µmole of methanol into formaldehyde per minute at pH 7.5 and at 25°C.
5.6. ammonium acetate (CH_3COONH_4, 99.5% pure - MW = 77.08g/mole)
5.7. pentane-2.4-dione ($C_5H_8O_2$ - MW = 100.12g/mole)
5.8. methanol ($CH_{20}H$, Analytical Reagent grade - MW = 32g/mole)
5.9. potassium dihydrogen phosphate (KH_2PO_4, 99% pure - MW = 136.06 g/mole)
5.10. disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$ 98.5% pure - MW = 178.05 g/mole)
5.11. distilled water
5.12. commercial enzyme preparation to be analysed

6. Solutions

6.1. Sodium acetate buffer 50 mM, pH 4.5

This consists of 2 solutions, A and B.

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6.1.1. Solution A: introduce 4.10 g of sodium acetate (5.3) into 1 liter of distilled water (5.11).

6.1.2. Solution B: introduce 2.8 ml of acetic acid (5.4) into 1 liter of distilled water (5.11).

6.1.3 Preparation of the sodium acetate buffer: mix 39.2% of solution A (6.1.1) + 60.8% of solution B (6.1.2),. Check that the pH equals 4.5 using a pH-meter (4.10). Maintain at 4°C

6.2. Citrus fruit pectin solution at 0.5% (p/v)

Introduce 0.5 g of citrus fruit pectin (5.1) into 100 ml of sodium acetate buffer (6.1) in a 100-ml graduated flask (4.9).

The solution must be prepared as needed.

6.3. Acetic acid solution 0.05 M

Introduce 0.283 5 ml of acetic acid (5.4) into 100 ml of distilled water (5.11), in a 100-ml graduated flask (4.8).

6.4. Ammonium acetate solution 2 M

Dissolve 15.4 g of ammonium acetate (5.6) in 100 ml of acetic acid (6.3), in a 100-ml graduated flask (4.9).

6.5. 2,4-Pentanedione 0.02 M

Introduce 40.8 µl 2,4-pentanedione (5.7) into 20 ml of ammonium acetate solution (6.4). The solution must be prepared as needed.

6.6. Sodium phosphate buffer (0.25 M; pH 7.5)

This consists of solutions A and B.

6.6.1. Solution A: introduce 34.015 g of potassium dihydrogen phosphate (5.9) into 1 liter of distilled water (5.11).

6.6.2. Solution B: introduce 44.5125 g of disodium hydrogen phosphate (5.10) into 1 liter of distilled water (5.11).

6.6.3. Preparation of the sodium phosphate buffer: mix 16.25 % of solution A (6.6.1) + 83.75% of solution B (6.6.2) to obtain a pH of 7.5.

Check the pH using a pH-meter (4.10).

Maintain at 4°C, for a maximum of one week

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6.7. Stock solution of methanol at 40 µg/ml

Introduce 5 µl of methanol (5.8) using a precision syringe (4.14) into 100 ml of sodium phosphate buffer (6.6) in a 100-ml graduated flask (4.9).

6.8. Alcohol oxydase at 1U/ml

Dilute alcohol oxydase of Pichia pastoris (5.5) in a phosphate buffer (6.6) in order to obtain a solution at 1U/ml. The solution must be prepared as needed.

7. Preparation of the standard solutions of methanol

The standard solutions are produced from 0 to 20 µg methanol as indicated in Table 1. They are made up from the stock solution of methanol (6.7.)

Table 1: standard solutions of methanol

Quantity of Methanol (µg)	0	5	10	15	20
Quantity of Methanol (µmole)	0	0.1563	0.3125	0.4688	0.625
Vol. stock solution (6.7.) (µl)	0	75	150	225	300
Vol. buffer (6.6.) (µl)	600	525	450	375	300

8. Preparation of the sample

It is important to homogenise the enzyme preparation before sampling, by upturning the container for example. The enzyme solution and the blanks have to be prepared at the time of use.

8.1. Enzyme solution with 1 g/l to be prepared just before use

Place 100 mg of commercial preparation (5.12) in a 100-ml graduated flask (4.9), make up to the mark with distilled water (5.11), and stir (4.19) in order to obtain a homogeneous mixture.

8.2. Blank denatured by heating to be prepared just before use

Place 10 ml of the enzyme solution at 1 g/l (8.1) in a 15-ml screw-top test tube (4.16),

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and immerse the test tube for 5 minutes in the water bath at 100°C (4.4). Cool and centrifuge for 5 min at 6500 g.

9. Procedure

9.1. Enzyme kinetics: The test tubes are prepared at least in duplicate.

In 5 x 15-ml test tubes (4.16) numbered from 1 to 5, placed in a rack (4.17) in a water bath at 30°C introduce:

100 µl of the enzyme solution at 1 g/l (8.1), using the precision syringe (4.13),

500 µl of the citrus fruit pectin solution (6.2) warmed beforehand at 30°C in a water bath, start the stop-watch (4.6).

After shaking (4.18), the test tubes are replaced in the water bath at 30°C (4.2):

- for 1 min. for test tube N°1
- for 2 min. for test tube N°2
- for 5 min. for test tube N°3
- for 10 min. for test tube N°4
- for 15 min. for test tube N°5

The reaction is stopped by placing each of the test tubes numbered from 1 to 5, immediately after they have been removed from the water bath at 30°C, in the water bath at 100°C (4.3) for 10 min.

The test tubes are then cooled under running cold water.

Note: the kinetic point at 10 min is used for the evaluation of the enzyme activity

9.2. Determination of methanol released

In a 15-ml screw-top test tube (4.16)

Add 1 ml of the alcohol oxydase solution (6.8) to the reaction medium (9.1), using the precision syringe (4.12), start the stop-watch (4.6).

After shaking (4.18), the test tube is placed in the water bath at 25°C (4.1) for 15 min.

Then add 2 ml of 0.02 M 2,4-pentanedione (6.5) using the precision syringe (4.11), start the stop-watch (4.6).

After shaking (4.18), the test tube is placed in the water bath at 60°C (4.3) for 15 min.

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The test tube is then cooled under running cold water.

Place the supernatant liquid in a cuvette (4.7).

Zero the spectrophotometer using distilled water.

Immediately measure the absorbance at 412 nm (4.15).

9.3. Blanks

Proceed as described in 9.1, replacing the enzyme solution at 1 g/l (8.1) by the blank denatured by heat (8.2). For each kinetic point, the enzymatic reaction of each blank is carried out at the same time as that of the enzyme solution.

9.4. Standard solutions

Proceed as described in 9.2, replacing the reaction mixture (9.1) by the various mixtures of the standard solutions of methanol from 0 to 20 µg (7).

10. Calculations

10.1. Determining the reaction 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 curve: the enzymatic activity is linear in time. Otherwise, the activity would be underestimated (Figure 1).

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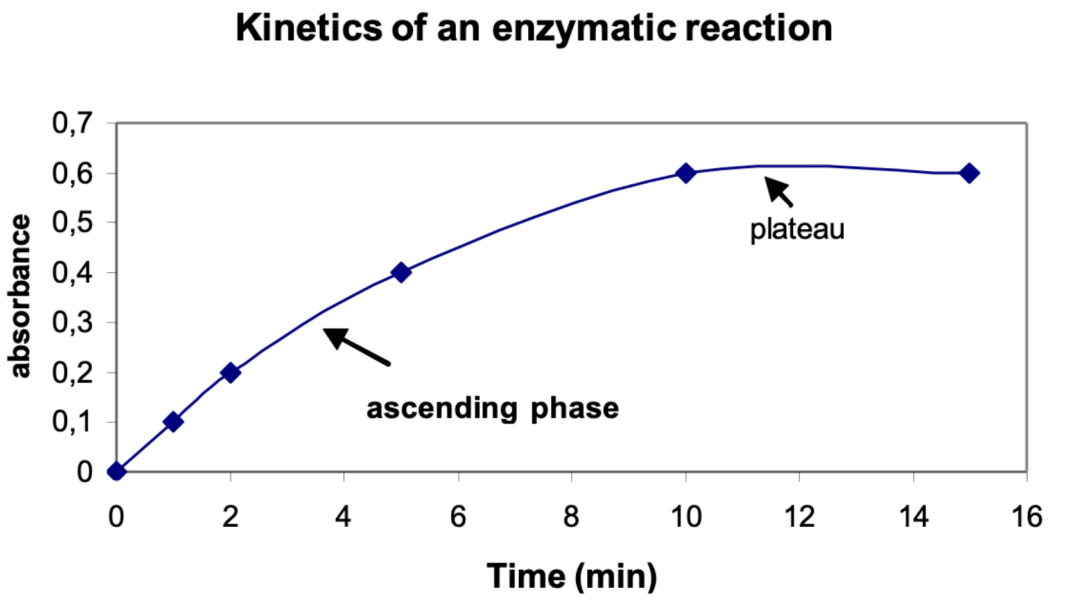

Figure 1: Kinetics of an enzymatic reaction

The kinetics are determined over 15 minutes. The activity concerned is measured at T=1 min T=2 min, T=5 min, T=10 min, T=15 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 enzyme 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 solutions of methanol (from 0 to 0.625 µmole) and whose Y-coordinates are the corresponding values of optical densities, obtained in 9.4. Then calculate the straight regression line (2) resulting from the linearity of the data of the graph.

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 methanol

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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 x (Q/T)/(VxC)

Where Q: quantity of methanol released in µmoles during time T (min)

V: quantity of enzyme solution introduced (ml), in this case 0.1 ml

C: concentration of the enzyme solution (g/l), in this case 1 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) x (1000/60)

11. Characteristics of the method

r =0.14

R = 0.112

Sr = 0.05

SR = 0.04

The intralaboratory repeatability of the method is estimated using the mean standard deviation of the absorbance values resulting from the same sampling of the enzymatic preparation, determined 5 times. In this way, for the pectin-methyl-esterase determination the mean standard deviation of the values is 0.05 with a percentage error of 5.46, in which the % error corresponds to:

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

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

The intralaboratory 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:

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- analysis of variance (the study of the probability of the occurrence of differences between samplings). Analysis of variance is a statistical method used to test the homogeneity hypothesis of a series of K averages. Performing the analysis of variance consists in determining if the "treatment" effect is "significant or not". The standard deviation of reproducibility given by this analysis of variance is 0.04.
- the power of the test for the first type of risk α (5%) – first type of risk α 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	Analysis of variance hypotheses	Probability	Power of test (□= 5%)	Newman-Keuls test (*)	Bonferroni test (**)
PME	Adhered to	0.00001	99%	Significant	Significant

Table 2: analysis of variance– 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 □ 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 □ 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 method of determination involves a probability of occurrence of a discrepancy in activity (between samplings) lower than 5%.

12. Bibliographical references

1. KLAVONS J.A., BENNET R.D., Determination of methanol using alcohol oxydase and its application to methyl ester content of pectins. J. Agr. Food. Chem, 1986. Vol 34,

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