COEI-1-ACTPME Determination of pectin methylesterase activity in enzymatic preparations ((Pectin Methyl-Esterase Activity (PME) (EC. 3.1.1.11 – CAS N° 9025-98-3)

General specifications

These enzymes are usually present within an complex enzymatic preparation. Unless otherwise stipulated, the specifications must comply with the resolution OIV/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 monography on Enzymatic preparation

The enzyme preparations containing such activity are produced by directed fermentations such as Aspergillus niger, *Aspergillus oryzae, Aspergillus sojae, Aspergillus Tubigensis, Aspergillus Awamori, Rhizopus oryzae and Trichoderma longibrachiatum (T.reesei)*

2. Scope /Applications

Reference is made to the International Code of Oenological Practices, OENO 11/2004; OENO 12/2004; OENO 13/2004; OENO 14/2004 and OENO 15/2004.

These enzyme activities are used to support grape maceration and grape juice extraction as well as to help the clarification of musts and wines and finally to improve their filterability.

Determination of Pectine methylesterase activity using methanol dosage

1. 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.

2. Equipment

2.1. water bath at 25°C
2.2. water bath at 30°C
2.3. water bath at 60°C
2.4. water bath at 100°C
2.5. 100-ml cylindrical flask
2.6. stop-watch
2.7. disposable spectrophotometer cuvettes with a 1-cm optical path length, for measurement in the visible spectrum
2.8. 1-L graduated flask
2.9. 100-ml graduated flask
2.10. pH-meter
2.11. 500-5000 µl precision syringe
2.12. 100-1000 µl precision syringe
2.13. 0-200 µl precision syringe
2.14. 0-20 µl precision syringe
2.15. spectrophotometer
2.16. 15-ml sealed glass screw-top test tubes
2.17. metal rack for 15 ml test tubes
2.18. Vortex-type mixer
2.19. magnetic stirrer

3. **Reagen**ts

3.1. citrus fruit pectin with a degree of esterification of 63-66%. (Pectins *ex-citrus:* Fluka, ref: 76280 as an example).
3.2. orange peel pectin esterase (Fluka; 20 U/mg, ref: 76286 as an example).
3.3. sodium acetate (CH_3COONa 99% pure - MW = 82g/mole)
3.4. acetic acid (CH_3COOH 96% pure - MW = 60 g/mole, density = 1.058)
3.5. 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.
3.6. ammonium acetate (CH_3COONH_4, 99.5% pure - MW = 77.08g/mole)
3.7. pentane-2.4-dione ($C_5H_8O_2$ - MW = 100.12g/mole)
3.8. methanol (CH_20H, Analytical Reagent grade - MW = 32g/mole)

3.9. potassium dihydrogen phosphate (KH_2PO_4, 99% pure - MW = 136.06 g/mole)

3.10. disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$ 98.5% pure - MW = 178.05 g/mole)

3.11. distilled water

3.12. commercial enzyme preparation to be analysed

4. **Solutio**ns

4.1. Sodium acetate buffer 50 mM, pH 4.5

This consists of 2 solutions, A and B.

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

4.1.2. 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

4.2. 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.

4.3. 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).

4.4. 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).

4.5. 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.

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

This consists of solutions A and B.

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

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

4.6.3. 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

4.7. 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).

4.8. 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.

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.)

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

Table 1: standard solutions of methanol

5. 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.

5.1. 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.

5.2. 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), 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.

6. Procedure

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

6.2. 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.

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).

6.3. 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.

6.4. 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).

7. Calculations

7.1. 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).

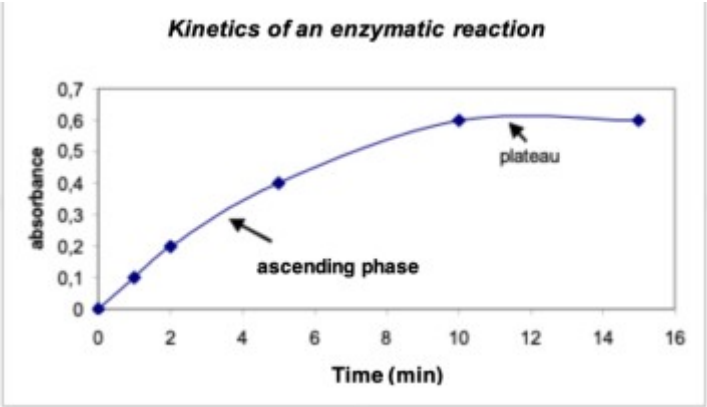


Figure 1: Kinetics of an enzymatic reaction

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).

7.2. 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.

7.3. 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 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

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

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:

$$\text{Activity in nkat/g} = (\text{activity in U/g}) \times (1000/60)$$

8. 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:

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

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:

- 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 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%.

9. Bibliographical references

- 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, p 597-599.
- Enzyme activities and their measurement – OIV Document, FV 1226, 2005

Determination of Pectinmethylesterase activity using acid based titration

1. Principle

The demethylation activity of the pectinmethylesterase results in the appearance of free carboxylic groups at the level of the galacturonic acids forming the chains. To determine the activity of pectinmethylesterase, the carboxyl groups can be titrated during the enzymatic hydrolysis with sodium hydroxide solution at constant temperature and constant pH-value.

2. Equipment and materials

- titration equipment (burette)
- temperature controlled heat plate and magnetic stirrer/magnetic stir bar
- pH meter
- glass cup, filled with water
- chronometer
- graduated flasks (different volume)
- beakers (preferably 50 mL)
- precision pipettes (different volume)

3. Chemicals and reagents

- Pectin; highly esterified; p.a. quality (Sigma P9135-100G); CAS 9000-69-5
- 0,01 M NaOH solution (Titrisol) p.a. quality; CAS 1310-73-2
- NaOH pellets p.a. quality ; CAS 1310-73-2

4. Preparation of soultions

4.1. 1 M NaOH

Dissolve 4 g NaOH in 100 mL H2O

4.2. substrate solution

As substrate solution 1 % Pectin in H2O, is used by solving 2.0 g Pectin very slowly in 150 ml H_2O. Subsequently the pH value is adjusted at pH 4.0 and at 40 °C with 1 M NaOH. The solution must be filled up to 200 mL exactly. Just before measuring, the pH-value should be controlled and adjusted again at pH 4,0, if necessary

4.3. enzymatic solution

The enzymatic solution consists of approximately 30 to 50 mg/L commercial enzyme preparation diluted in cold water. This solution should be prepared directly before using.

4.4. 0.01M NaOH

This precast solution should be diluted according to the description of the producer.

4.5. Performance of enzyme activity determination

20 ml of substrate solution are put in a beaker (magnetic stirrer is added) on the temperature controlled heat plate in a glass cup, which is filled with water heated up to 40 °C. The pH electrode is put in substrate solution. It is necessary to have a control and maybe a new setting up of the pH-value at 40 °C before starting the analysis. Then 0.1 ml of the enzymatic solution is added. Exactly at this time the chronometer is started. During the analysis the pH value must be measured and the sample has to be titrated up to pH 4.0 with 0.01 M NaOH for 10 minutes at 40 °C. After 10 min the analysis is stopped and the consumption of 0.01 M NaOH is read off.

The consumption of 0,01 M NaOH should amount to values between 3,5 mL and 8,5 mL. Otherwise it is recommended to dilute or concentrate the enzymatic solution.

5. Calculation of the enzymatic activity

Enzymatic activity is calculated by using following formula:

Activity $(U/mg) = n/(t * v * c)$

Activity $(nkat/g) = (Activity(U/mg) * 1000/60) * 1000$

- n = consumption of 0.01 M NaOH in µmol
- t = time in min (in this case 10 min)
- v = quantity of enzymatic solution introduced in ml (=0.1 ml)
- c = concentration of the enzymatic solution in g/L

Validation of the acid based titration to determine the activity of Pectin methylesterase

The mean value of the standard deviation was determined of 8 different enzymes.

Each enzyme was analysed 6 times.

Mean value of the standard deviations of the different enzymes = 3.91 %

	Enzyme 1 40 mg/ml	Enzyme 2 40 mg/ml	Enzyme 3 40 mg/ml	Enzyme 4 40 mg/ml	Enzyme 5 40 mg/ml	Enzyme 6 40 mg/ml	Enzyme 7 30 mg/ml	Enzyme 8 50 mg/ml	Enzyme 8 30 mg/ml
Mean Value (nkat/g)	14527.7	19291.7	12756.8	9534.7	9444.5	18577.8	31591.7	10888.9	9446.5
Standard Deviation (nkat/g)	282.3	449.5	366.4	227.4	272.3	145.6	540.9	944.4	1096.1
Standard Deviation %	**1.9**	**2.3**	**2.9**	**2.4**	**2.9**	**0.8**	**1.7**	**8.7**	**11.6**
$s^2(r)$	66410	168402	111863	43097	61786	17654	243773	743210	1001244
s (r)	257.7	410.4	334.5	207.6	248.6	132.9	493.7	862.1	1000.6
Repeatability r (nkat/g)	729.3	1161.3	946.5	581.5	703.4	376.0	1397.3	2439.7	2831.8

Validation of the acid based titration to determine the activity of PME

Enzyme	Concentration	U/mg	nkat/g
Enzyme 1	40 mg/ml	0.89	14833
Enzyme 1	40 mg/ml	0.89	14750
Enzyme 1	40 mg/ml	0.88	14667
Enzyme 1	40 mg/ml	0.85	14083
Enzyme 1	40 mg/ml	0.87	14500
Enzyme 1	40 mg/ml	0.86	14333

Enzyme 1 40 mg/ml			(X-MW)^2
mean value (nkat/g)	14527.7		93228.4
standard deviation (nkat/g)	282.30		49432.1
standard deviation %	1.9		19413.8
variance	3.8		197728.4
$s^2(r)$	66410.6		765.4
s(r)	257.7		37895.1
r (nkat/g) repeatability	729.3	sum	398463.3

Enzyme	Concentration	U/mg	nkat/g
Enzyme 2	40 mg/ml	1.185	19750
Enzyme 2	40 mg/ml	1.155	19250
Enzyme 2	40 mg/ml	1.130	18833
Enzyme 2	40 mg/ml	1.125	18750
Enzyme 2	40 mg/ml	1.190	19833
Enzyme 2	40 mg/ml	1.160	19333

Enzyme 2 40 mg/ml			(X-MW)^2
mean value (nkat/g)	19291.7		210069.4
standard deviation (nkat/g)	449.54		1736.1
standard deviation %	2.3		210069.4
$s^2(r)$	168402.8		293402.8
s(r)	410.4		293402.8
r (nkat/g) repeatability	1161.3		1736.1
		sum	1010416.7

Enzyme	Concentration	U/mg	nkat/g
Enzyme 3	40 mg/ml	0.78	13042
Enzyme 3	40 mg/ml	0.79	13208
Enzyme 3	40 mg/ml	0.76	12708
Enzyme 3	40 mg/ml	0.76	12583
Enzyme 3	40 mg/ml	0.77	12833
Enzyme 3	40 mg/ml	0.73	12167

Enzyme 3 40 mg/ml			(X-MW)^2
mean value (nkat/g)	12756.8		81320.0
standard deviation (nkat/g)	366.38		203551.4
standard deviation %	2.9		2384.7
$s^2(r)$	111863.1		30218.0
s(r)	334.5		5801.4
r (nkat/g) repeatability	946.5		347903.4
		sum	671178.8

Enzyme	Concentration	U/mg	nkat/g
Enzyme 4	40 mg/ml	0.57	9500
Enzyme 4	40 mg/ml	0.59	9875
Enzyme 4	40 mg/ml	0.56	9333
Enzyme 4	40 mg/ml	0.56	9250
Enzyme 4	40 mg/ml	0.58	9583
Enzyme 4	40 mg/ml	0.58	9667

Enzyme 4 40 mg/ml			(X-MW)^2
mean value (nkat/g)	9534.67		1201.8
standard deviation (nkat/g)	227.41		115826.8
standard deviation %	2.4		40669.4
$s^2(r)$	43096.9		81035.1
s(r)	207.6		2336.1
r (nkat/g) repeatability	587.5		17512.1
		sum	258581.3

Enzyme	Concentration	U/mg	nkat/g
Enzyme 5	40 mg/ml	0.55	9167
Enzyme 5	40 mg/ml	0.59	9792
Enzyme 5	40 mg/ml	0.55	9083
Enzyme 5	40 mg/ml	0.57	9458
Enzyme 5	40 mg/ml	0.57	9542
Enzyme 5	40 mg/ml	0.58	9625

Enzyme 5 40 mg/ml			(X-MW)^2
mean value (nkat/g)	9444.5		77006.3
standard deviation (nkat/g)	272.29		120756.3
standard deviation %	2.9		130682.3
$s^2(r)$	61785.6		182.3
s(r)	248.6		9506.3
r (nkat/g) repeatability	703.4		32580.3
		sum	370713.5

Enzyme	Concentration	U/mg	nkat/g
Enzyme 6	40 mg/ml	1.105	18417
Enzyme 6	40 mg/ml	1.118	18633
Enzyme 6	40 mg/ml	1.125	18750
Enzyme 6	40 mg/ml	1.105	18417
Enzyme 6	40 mg/ml	1.112	18533
Enzyme 6	40 mg/ml	1.123	18717

Enzyme 6 40 mg/ml			(X-MW)^2
mean value (nkat/g)	18577.8		25956.8
standard deviation (nkat/g)	145.55		3086.4
standard deviation %	0.8		29660.5
$s^2(r)$	17654.3		25956.8
s(r)	132.9		1975.3
r (nkat/g) repeatability	376.0		19290.1
		sum	105925.9

Enzyme	Concentration	U/mg	nkat/g
Enzyme 7	30 mg/ml	1.920	32000
Enzyme 7	30 mg/ml	1.947	32450
Enzyme 7	30 mg/ml	1.873	31217
Enzyme 7	30 mg/ml	1.860	31000
Enzyme 7	30 mg/ml	1.893	31550
Enzyme 7	30 mg/ml	1.880	31333

Enzyme 7 30 mg/ml			(X-MW)^2
mean value (nkat/g)	31591.7		166736.1
standard deviation (nkat/g)	540.86		736736.1
standard deviation %	1.7		140625.0
$s^2(r)$	243773.1		350069.4
s(r)	493.7		1736.1
r (nkat/g) repeatability	1397.3		66736.1
		sum	1462638.9

Enzyme	Concentration	U/mg	nkat/g
Enzyme 8	50 mg/ml	0.578	9633
Enzyme 8	50 mg/ml	0.682	11367
Enzyme 8	50 mg/ml	0.706	11767
Enzyme 8	50 mg/ml	0.712	11867
Enzyme 8	50 mg/ml	0.596	9933
Enzyme 8	50 mg/ml	0.646	10767

Enzyme 8 50 mg/ml			(X-MW)^2
mean value (nkat/g)	10888.9		1576419.8
standard deviation (nkat/g)	944.38		228271.6
standard deviation %	8.7		770493.8
$s^2(r)$	743209.9		956049.4
s(r)	862.1		913086.4
r (nkat/g) repeatability	2439.7		14938.3
		sum	4459259.3

Enzyme	Concentration	U/mg	nkat/g
Enzyme 8	30 mg/ml	0.69	11444
Enzyme 8	30 mg/ml	0.067	8667
Enzyme 8	30 mg/ml	0.063	8889
Enzyme 8	30 mg/ml	0.065	8429
Enzyme 8	30 mg/ml	0.07	9625
Enzyme 8	30 mg/ml	0.067	9625

Enzyme 8 30 mg/ml			(X-MW)^2
mean value (nkat/g)	9446.5		3990006.3
standard deviation (nkat/g)	1096.13		607620.3
standard deviation %	11.6		310806.3
$s^2(r)$	1001243.9		1035306.3
s(r)	1000.6		31862.3
r (nkat/g) repeatability	2831.8		31862.3
		sum	6007463.5

mean value of the standard deviations %	**3.91**