COEI-1-ACTPGA Determination of polygalacturonase activity in enzymatic preparations (endo- and exo-polygalacturonase activities (PG)

(EC. 3.2.1.15 – CAS N° 9032-75-1)

General specifications

These enzymes are generally present among other activities, within an enzyme complex, but may also be available in purified form, either by purification from complex pectinases or directly produced with Genetically Modified Microorganisms. 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 “Sources of enzymes and fermentation environment” of the general monograph on enzymatic preparations.

The enzyme preparations containing such activity are produced by directed fermentations such as *Aspergillus niger, Rhizopus oryzae and Trichoderma reesei or longibrachiatum*

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 contribute to the effectiveness of grape maceration and grape juice extraction as well as to help the clarification of musts and wines and finally to improve their filterability.

Methods

1. Methods 1

2. Scope

The method of determination was developed using a commercially available polygalacturonase. The conditions and the method were developed for application to

the commercial enzyme preparations such as those found on the oenological market.

3. Principle

Polygalacturonases cut pectin chains with a low degree of methylation and thus release the galacturonic acids forming the pectin located at the ends of the chain. Once released, the galacturonic acids are determined by the Nelson method (1944). In an alkaline medium, the pseudo aldehyde group of sugars reduces the cupric ions Cu^{2+}. The latter react with the arsenomolybdate reagent to produce a blue colour, whose absorbance, measured at 520 nm, varies linearly with the concentration in monosaccharides (between 0 and 250 µg/mL).

4. Equipment

4.1. magnetic stirrer with hot-plate
4.2. water bath at 40°C
4.3. water bath at 100°C
4.4. 100-ml beaker
4.5. centrifuge capable of housing 15-mL glass test tubes
4.6. stop-watch
4.7. 100-ml graduated flask
4.7.1. 500-ml graduated flask
4.8. 200- µl precision syringe
4.8.1. 1-ml precision syringe
4.9. 10-ml straight pipette graduated to 1/10 mL
4.10. spectrophotometer
4.11. 15-mL glass test tubes
4.12. Vortex-type mixer
4.13. 500-mL amber glass bottle
4.14. room at 4°C
4.15. drying oven at 37°C
4.16. cotton-wool
4.17. brown paper
4.18. pH-meter
4.19. metal rack for 15-mL test tubes
4.20. disposable spectrophotometer cuvettes with a 1-cm optical path length, for

measurement in the visible spectrum.

5. Reagents

5.1. sodium acetate (CH_3COONa 99% pure - MW = 82g/mole)

5.2. acetic acid (CH_3COOH 96% pure - MW = 60 g/mole, density = 1.058)

5.3. polygalacturonic acid 85% pure. "Polygalacturonic acid sodium salt" from citrus fruit (Sigma, P3 850) is an example.

5.4. anhydrous sodium sulphate (Na_2SO_4 99.5% pure - MW = 142 g/mole)

5.5. anhydrous sodium carbonate (Na_2CO_3 99.5% pure - MW = 105.99 g/mole)

5.6. sodium potassium tartrate ($KNaC_4H_2O_6.4H_2O$ 99% pure - MW = 282.2 g/mole)

5.7. anhydrous sodium bicarbonate ($NaHCO_3$ 98% pure - MW = 84.0 1 g/mole)

5.8. copper sulfate penta-hydrated ($CuSO_4.5H_2O$ 99% pure - MW = 249.68 g/mole)

5.9. concentrated sulphuric acid (H_2SO_4 98% pure)

5.10. ammonium heptamolybdate ($(NH_4)_6MO_7O_{24}.4H_2O$ 99% pure - MW = 1235.86 g/mole)

5.11. sodium hydrogenoarsenate ($Na_2HA_sO_4.7H_2O$ 98.5% pure - MW = 3 12.02 g/mole). **Given the toxicity of this product, special attention must be paid during manipulation. Waste material must be treated in an appropriate manner.**

5.12. D-galacturonic acid ($C_5H_{10}O_7.H_2O$ - MW: 2 12.16 g/mole)

5.13. distilled water

5.14. commercial enzyme preparation to be analysed

6. Solutions

1. Reagents of the oxidizing solution

These reagents have to be prepared first, taking into account the 24-hour lead-time for solution D.

6.1.1. Solution A: Place successively in a 100-mL beaker (4.4):

- 20 g of anhydrous sodium sulphate (5.4)
- 2.5 g of anhydrous sodium carbonate (5.5)
- 2.5 g of sodium potassium tartrate (5.6)
- 2 g of anhydrous sodium bicarbonate (5.7)

Dissolve in 80 ml of distilled water (5.13). Heat (4.1) until dissolution and transfer into a 100-ml graduated flask (4.7). Make up to the mark with distilled water (5.13). Maintain at 37°C (4.15); if a deposit forms, filter on a folded filter.

6.1.2. Solution B:

Dissolve 15 g of copper sulfate pentahydrate (5.8) in 100 mL of distilled water (5.13) and add a drop of concentrated sulphuric acid (5.9). Maintain at 4°C.

6.1.3. Solution C:

This solution is prepared just before use in order to have a satisfactory proportionality between the depth of colour and the quantity of glucose by mixing 1 mL of solution B (6.1.2) with 24 mL of solution A (6.1.1).

6.1.4. Solution D:

In a 500-mL graduated flask (4.7.1), dissolve 25 g of ammonium molybdate (5.10) in 400 mL of water (5.13). Add 25 ml of concentrated sulphuric acid (5.9) (cooled under running cold water).

In a 100-mL beaker (4.4) dissolve 3 g of sodium arsenate (5.11) in 25 mL of water (5.13) and transfer quantitatively into the 500-mL graduated flask (4.7.1) containing the ammonium molybdate (5.10).

Make up to the mark with water (5.13) to have a final volume of 500 mL.

Place at 37°C (4.15) for 24 hours then maintain at 4°C (4.14) in a 500 mL amber glass bottle (4.13).

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

This consists of solutions A and B.

6.2.1. Solution A: sodium acetate 0.1 M: dissolve 0.5 g of sodium acetate (5.1) in 60 mL of distilled water (5.13)

6.2.2. Solution B: acetic acid 0.1 M: dilute 1 mL of acetic acid (5.2) with 175 mL of distilled water (5.13)

6.2.3. Preparation of the sodium acetate buffer: mix 23.9 ml of solution A (6.2.1) + 76.1 ml of solution B (6.2.2).

Check the pH of the buffer using a pH-meter (4.18).

The solution must be maintained at 4°C (4.14).

6.3. Polygalacturonic acid solution at 0.4 % (p/v)

In a 100 mL graduated flask (4.7) dissolve 0.4 g of polygalacturonic acid (5.3) in 100 mL of sodium acetate buffer (6.2).

The solution must be prepared just before use.

6.4. Stock solution of D-galacturonic acid at 250 µg/ml

In a 100 mL graduated flask (4.7), dissolve 0.0250 g of D-galacturonic acid (5.12) in distilled water (5.13) and make up to 100 mL.

7. Preparation of the standard solutions of D-galacturonic acid

The standard range is produced from 0 to 250 µg/mL, according to table 1.

Table 1: standard solutions of D-galacturonic acid

Galacturonic acid (µg/mL) 0	25	50	100	150	200	250
Galacturonic acid (µmole/mL)0	0.118	0.236	0.471	0.707	0.943	1.178
Vol. (µl) stock solution (6.4)0	100	200	400	600	800	1000
Vol. (µl) distilled water (5.13) 1000	900	800	600	400	200	0

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 will have to be prepared at the time of use.

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

Place 100 mg of commercial preparation (5.14) in a 100-ml graduated flask (4.7), make up with distilled water (5.13), and stir 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 test tube (4.11), plug with cotton wool (4.16) covered with brown paper (4.17) and immerse the test tube for 5 minutes in the water bath at 100°C (4.3). Cool and centrifuge 5 min at 6500 g.

9. Procedure

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

In 5 x 15-ml test tubes (4.11) numbered from 1 to 5, placed in a rack (4.19) in a water bath at 40°C, introduce

- 200 µl of the enzyme solution at 1 g/l (8.1), using the precision syringe (4.8),

- 400 µl of distilled water (5.13), using the precision syringe (4.8.1),
- 600 µl of the polygalacturonic acid (6.3) warmed beforehand at 40°C in a water bath, start the stop-watch (4.6).

After shaking (4.12), the test tubes plugged with cotton-wool (4.16) and brown paper (4.17) are replaced in the water bath at 40°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 40°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 reducing substances released

In a 15-mL test tube (4.11)

Place 1 mL of the reaction medium (9.1) using the precision syringe (4.8.3)

Add 1 mL of solution C (6.1.3) using the precision syringe (4.8.3)

After shaking (4.12), the test tube is placed in the water bath at 100°C (4.3) for 10 min. The test tube is then cooled under running cold water.

Add 1 mL of solution D (6.1.4)

Add 9.5 ml of water (5.13) using the straight 10-mL pipette (4.9)

Wait 10 min. for the colour to stabilise.

Centrifuge (4.5) each test tube at 2430 g for 10 min.

Place the supernatant liquid in a cuvette (4.20).

Zero the spectrophotometer using distilled water

Immediately measure the absorbance at 520 nm, using a spectrophotometer (4.10).

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 D-galacturonic acid from 0 to 250 µg/mL (7).

10. Calculation

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).

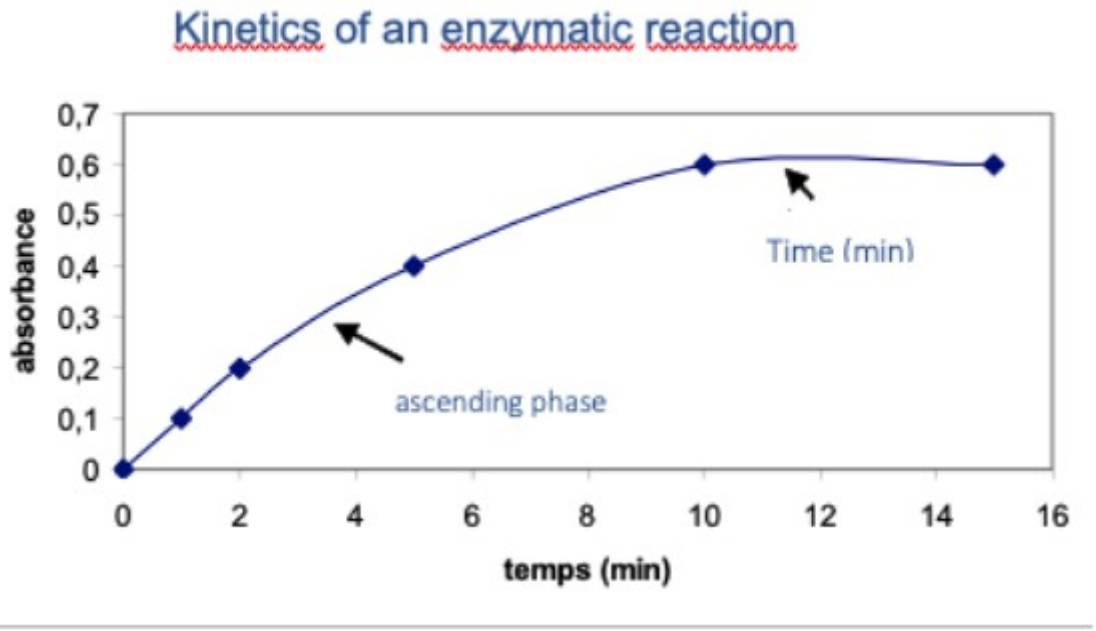

Figure 1: Kinetics of an enzymatic reaction

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 D-galacturonic acid (from 0 to 0.589 µmole/mL) and whose Y-coordinates are the corresponding values of optical densities, obtained in 9.4. Then calculate the straight regression slope 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 D-galacturonic acid 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 D-galacturonic acid released in µmoles during time T (min)
- V: quantity of enzyme solution introduced (mL), in this case 0.2 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:

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

11. Characteristics of the method

r	0.084
R	0.056
Sr	0.03
SR	0.02

The intralaboratory repeatability of the method is estimated using the mean standard deviation of the absorbance values resulting from the same sampling of the enzyme preparation, determined 5 times. In this way, to analyse the polygalacturonase the mean standard deviation of the values is 0.03 with a percentage error of 3.78, in which

the % error corresponds to:

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

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

The intralaboratory reproducibility tests were carried out using 2 enzyme 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 0.02.
- 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 (**)
PG	Treatment* block interaction	0.0256	77%	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. Bibliography

- NELSON N, A photometric adaptation of the SOMOGYI method for the determination of glucose. The may Institute for medical research of the Jewish hospital, 1944. p 375-380.
- Enzyme activities and their measurement – OIV Document, FV 1226, 2005

Methods 2: Determination of Polygalacturonase activity with cyanoacetamide

1. Principle

Polygalacturonases cut the principal pectin chains (homogalacturonan domain) with a low degree of methylation. This enzyme activity leads to the release of galacturonic acids along with the homogalacturonan oligomers. Therefore the reducing ends are released. This ultraviolet method with cyanoacetamide, based on KNOEVENAGEL reaction, which means the condensation between an active methylen group and a carbonyl group in a strongly alkaline medium, is existing to find out the activity of various enzymes amongst others of polygalacturonase. It has been developed for the determination of the enzymatic degradation of polysaccharides through an endo- and exo- mechanism that generates reducing monosaccharides.

2. Equipment and materials

- Spectrophotometer
- quartz cuvette (λ=274 nm, optical path length 1 cm)
- analytical scale
- magnetic stirrer and stir bar
- water-bath (40°C; 100°C)
- chronometer
- graduated flasks (different volume)
- beakers (different volume)

- precision pipettes (different volume)
- spectrophotometer
- glass tubes (closable)
- vortex mixer

3. Chemicals and reagents

- polygalacturonic acid, ~95 % enzymatic (CAS 25990-10-7)
- pH 4.0 Na-citrate/HCl buffer, 1.06 g/cm^3 (Titrisol), p.a. quality
- pH 9.0 H_3BO_3/KCl/NaOH buffer ≈0.05 M/≈0.05 M/≈0.022M (Titrisol), p.a. quality
- cyanoacetamide, ≥ 98 %, purum (CAS 107-91-5)
- D-galacturonic acid monohydrate ≥ 97 % (CAS 91510-62-2)

4. Preparation of solutions

4.1. Stock solution of D-galacturonic acid (250 µg/mL)

Dissolve 0,025 g of D-galacturonic acid in 100 mL H_2O.

4.2. 1 % cyanoacetamide solution

Dissolve 1 g of cyanoacetamide in 100 mL H_2O

4.3. Borate buffer (pH 9.0)

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

4.4. Na-citrate/HCl buffer (pH 4.0)

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

4.5. Polygalacturonic acid solution

Stirring constantly dissolve polygalacturonic acid very slowly in the concentration of 5 g/l in Na-citrat/HCl buffer (pH 4.0)

5. Performance of enzyme activity determination

5.1. Calibration curve and procedure

The standard range is produced from 0 µg/mL to 250 µg/mL of D-galacturonic acid. Use stock solution for dilution.

D-galacturonic acid monohydrate µg/mL	0	25	50	100	150	200	250
D-galacturonic acid monohydrate µmol/mL	0	0.118	0.236	0.471	0.707	0.943	1.178
Stock solution µL	0	100	200	400	600	800	1000
H_2O µL	1000	900	800	600	400	200	0

Cyanoacetamide assay: 1mL of D-galacturonic acid and 2 mL borate buffer (pH 9) and.1 mL of 1 % cyanoacetamide solution are mixed. After incubation in a test tube at 100°C for 10 min, the solution is cooled down in a cold water bath. Then the absorbance must be measured at 274 nm immediately. The photometer must be set to zero with water.

For calculation the intersection point of the regression line must be set to zero.

5.2. Enzymatic hydrolysis and procedure of the sample

For the enzymatic hydrolysis of polygalacturonic acid 10 mL of polygalacturonic acid solution must be heated at 40°C in a closable glass tube. Then 0,01 g of the sample is added and the mixture must be incubated at 40°C. After exactly 5 min and exactly 10 min, 500 µL are removed from the reaction mixture and directly heated up to 100°C in preheated test tubes for 10 min. Afterwards this 500 µL are diluted with water to a total volume of 25 mL.

For analysing the blank the same concentration of enzyme in polygalacturonic acid is heated up to 100 °C for 10 min (the polygalacturonic acid solution must be heated at 100°C before adding the enzyme!). In case of cloudiness the solution should be centrifuged at 5000 rpm for 5 min. Then the blank must also be incubated at 40°C. 500 µL of the blank solution are removed after 5 min and also placed in the water bath at 100°C for 10 min. Afterwards this 500 µL are diluted with water to a total volume of 25 mL.

Cyanoacetamide assay: 1 mL of the diluted solution and 1 mL of 1 % cyanoacetamide solution are added to 2 mL borate buffer (4.3.). After incubation in a test tube at 100°C for 10 min, the solution must be cooled down in a cold water bath. Then the absorbance must be measured at 274 nm immediately.

6. Calculation of the enzymatic activity

Enzymatic activity is calculated by relating the absorbance value and the quantity of

product formed using a standard range with the formula:

Activity (U/g) = q/ (t*c*F)

Activity (nkat/g) = q/ (t*c*F) *(1000/60)

- q = quantity of galacturonic acid in µmol/mL
- t = time in min
- c = concentration of the enzymatic solution in g/L (= 0.01 g/L) pro 10 mL substrat
- F = correction factor of the volume (=2)

7. Literature

Bach E. and Schollmeyer E. (1992): An Ultraviolett-Spectrophotometric Method with 2-Cyanoacetamide for the Determination of the Enzymatic Degradation of Reducing Polysaccharides. Anal. Biochem. 203, 335-339.

8. Intra-laboratory validation of the determination of the activity of Polygalacturonase with 2- Cyanoacetamide

The mean value of the standard deviation was determined of 6 different enzymes.

Each enzyme was analysed 6 times.

Mean value of the standard deviations of the different enzymes = 6,93 %

	Enzyme 1 5 min	Enzyme 2 5 min	Enzyme 3 5 min	Enzyme 4 5 min	Enzyme 5 5 min	Enzyme 6 5 min	Enzyme 4 10 min	Enzyme 5 10 min	Enzyme 6 10 min
Mean Value (nkat/g)	7583.9	3896.4	10445.8	8751.7	16894.4	16153.1	8532.5	11608.9	14436.1
Standard Deviation (nkat/g)	1195.6	367.1	445.3	420.4	631.4	908.7	246.48	656.3	1012.3
Standard Deviation %	15.8	9.4	4.3	4.8	3.7	5.6	2.9	5.7	7.0
$s^2(r)$	1191221	112292	165238	147264	332227	688096	50628	358948	853983
s (r)	1091.4	335.1	406.5	383.7	576.4	829.5	225.0	599.1	924.1
Repeatability r (nkat/g)	3088.7	948.3	1150.4	1086.0	1631.2	2347.5	636.8	1695.5	2615.2

Intra-laboratory validation of the determination of the activity of PG with 2-Cyanoacetamide

Enzyme	Absorbance 5 min	Concentration (mg/ml)	U/g	nkat/g
Enzyme 1	0.1698	0.01	389.2	6487
Enzyme 1	0.2278	0.01	593.6	9893
Enzyme 1	0.1855	0.01	444.5	7408
Enzyme 1	0.1815	0.01	430.4	7173
Enzyme 1	0.1887	0.01	455.9	7598
Enzyme 1	0.1776	0.01	416.6	6943

Enzyme 1; 5 min	
mean value (nkat/g)	7583.9
standard deviation (nkat/g)	1195.60
standard deviation %	15.77
Variance	248.5
$s^2(r)$	1191221.0
s(r)	1091.4
r (nkat/g) repeatability	3088.7

(X-MW)^2
1203896.6
5333533.6
30819.8
168555.9
208.6
410311.4
sum 7147325.9

Enzyme	Absorbance 5 min	Concentration (mg/ml)	U/g	nkat/g
Enzyme 2	0.0898	0.01	215.2	3587
Enzyme 2	0.0898	0.01	215.3	3588
Enzyme 2	0.0897	0.01	214.5	3575
Enzyme 2	0.09	0.01	245.2	4087
Enzyme 2	0.0954	0.01	245.6	4093
Enzyme 2	0.0971	0.01	266.9	4448

Enzyme 2; 5 min	
mean value (nkat/g)	3896.4
standard deviation (nkat/g)	367.08
standard deviation %	9.42
Variance	88.76
$s^2(r)$	112292.05
s(r)	335.10
r (nkat/g) repeatability	948.33

(X-MW)^2
95927.9
94898.2
103290.8
36205.6
38787.1
304642.7
sum 673752.3

Enzyme	Absorbance 5 min	Concentration (mg/ml)	U/g	nkat/g
Enzyme 3	0.4077	0.01	613.4	10223
Enzyme 3	0.3937	0.01	588.8	9813
Enzyme 3	0.4201	0.01	635.3	10588
Enzyme 3	0.4095	0.01	616.6	10277
Enzyme 3	0.4381	0.01	666.9	11115
Enzyme 3	0.4225	0.01	639.5	10658

Enzyme 3; 5 min	
mean value (nkat/g)	10445.83
standard deviation (nkat/g)	445.29
standard deviation %	4.26
Variance	18.2
$s^2(r)$	165237.7
s(r)	406.5
r (nkat/g) repeatability	1150.4

(X-MW)^2
49506.3
400056.3
20306.3
28617.4
447784.0
45156.3
sum 991426.4

Enzyme	Absorbance 5 min	Concentration (mg/ml)	U/g	nkat/g
Enzyme 4	0.2032	0.01	530.4	8840
Enzyme 4	0.19614	0.01	505.5	8425
Enzyme 4	0.21	0.01	555.9	9265
Enzyme 4	0.19188	0.01	490.5	8175
Enzyme 4	0.20858	0.01	549.3	9155
Enzyme 4	0.3448	0.01	519	8650

Enzyme 4; 5 min	
mean value (nkat/g)	8751.7
standard deviation (nkat/g)	420.38
standard deviation %	4.80
Variance	23.1
$s^2(r)$	147263.9
s(r)	383.7
r (nkat/g) repeatability	1066.0

(X-MW)^2
7802.8
106711.1
263511.1
332544.4
162677.8
10336.1
sum 883583.3

Enzyme	Absorbance 5 min	Concentration (mg/ml)	U/g	nkat/g
Enzyme 5	0.35063	0.01	978.1	16302
Enzyme 5	0.35329	0.01	987.5	16458
Enzyme 5	0.3812	0.01	1085.7	18095
Enzyme 5	0.35979	0.01	1010.4	16840
Enzyme 5	0.35941	0.01	1009.1	16818
Enzyme 5	0.4559	0.01	1011.2	16853

Enzyme 5; 5 min	
mean value (nkat/g)	16894.4
standard deviation (nkat/g)	631.40
standard deviation %	3.74
Variance	14.0
$s^2(r)$	332226.5
s(r)	576.4
r (nkat/g) repeatability	1631.2

(X-MW)^2
351385.5
190192.9
1441333.6
2964.2
5792.9
1690.1
sum 1993359.3

Enzyme	Absorbance 5 min	Concentration (mg/ml)	U/g	nkat/g
Enzyme 6	0.30006	0.01	888.5	14808
Enzyme 6	0.3108	0.01	926.2	15437
Enzyme 6	0.3348	0.01	1010.9	16848
Enzyme 6	0.3391	0.01	1025.9	17098
Enzyme 6	0.3195	0.01	957	15950
Enzyme 6	0.5370	0.01	1006.6	16777

Enzyme 6; 5 min	
mean value (nkat/g)	16153.1
standard deviation (nkat/g)	908.69
standard deviation %	5.63
Variance	31.6
$s^2(r)$	688095.8
s(r)	829.5
r (nkat/g) repeatability	2347.5

(X-MW)^2	
1808277.9	
513213.0	
483411.2	
893550.1	
41231.6	
388890.8	
sum	4128574.5

Enzyme	Absorbance 10 min	Concentration (mg/ml)	U/g	nkat/g
Enzyme 4	0.3355	0.01	498	8300
Enzyme 4	0.3569	0.01	535.8	8930
Enzyme 4	0.3340	0.01	495.4	8257
Enzyme 4	0.3420	0.01	509.5	8492
Enzyme 4	0.3472	0.01	518.6	8643
Enzyme 4	0.3448	0.01	514.4	8573

Enzyme 4; 10 min	
mean value (nkat/g)	8532.5
standard deviation (nkat/g)	246.48
standard deviation %	2.89
Variance	8.3
$s^2(r)$	50627.5
s(r)	225.0
r (nkat/g) repeatability	636.8

(X-MW)^2	
54056.3	
158006.3	
76084.0	
1667.4	
12284.0	
1667.4	
sum	303765.3

Enzyme	Absorbance 10 min	Concentration (mg/ml)	U/g	nkat/g
Enzyme 5	0.43542	0.01	638.3	10638
Enzyme 5	0.49384	0.01	741.2	12353
Enzyme 5	0.4712	0.01	701.4	11690
Enzyme 5	0.49213	0.01	738.2	12303
Enzyme 5	0.46232	0.01	685.7	11428
Enzyme 5	0.4559	0.01	674.4	11240

Enzyme 5; 10 min	
mean value (nkat/g)	11608.9
standard deviation (nkat/g)	656.31
standard deviation %	5.65
Variance	32.0
$s^2(r)$	358947.8
s(r)	599.1
r (nkat/g) repeatability	1695.5

(X-MW)^2	
941978.1	
554197.5	
6579.0	
482253.1	
32600.3	
136079.0	
sum	2153687.0

Enzyme	Absorbance 10 min	Concentration (mg/ml)	U/g	nkat/g
Enzyme 6	0.60886	0.01	987.9	16465
Enzyme 6	0.5221	0.01	835.1	13918
Enzyme 6	0.5180	0.01	828.0	13800
Enzyme 6	0.52344	0.01	837.5	13958
Enzyme 6	0.52895	0.01	847.2	14120
Enzyme 6	0.537	0.01	861.3	14355

Enzyme 6; 10 min	
mean value (nkat/g)	14436.1
standard deviation (nkat/g)	1012.31
standard deviation %	7.01
Variance	49.2
$s^2(r)$	853983.0
s(r)	924.1
r (nkat/g) repeatability	2615.2

(X-MW)^2	
4116390.1	
268093.8	
404637.3	
228271.6	
99926.2	
6579.0	
sum	5123898.1

mean value of the standard deviations %	**6.93**