RESOLUTION OIV-OENO 364-2012

DETERMINATION OF POLYGALACTURONASE ACTIVITY IN ENZYMATIC PREPARATIONS (COMPLEMENT TO RESOLUTION 10-2008)

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,

Considering the works of the group of experts Specifications of Oenological Products,

Considering the resolution OENO 10/2008 adopted in 2008 concerning polygalacturonase

HAS HEREBY DECIDED to complete the monograph on the determination of Polygalacturonase activity OENO 10/2008 published in the international Oenological Codex by the following method:

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 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 11/04; 12/04; 13/04; 14/04 and 15/04.

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.

Certified in conformity
Izmir, 22nd June 2012
The General Director of the OIV
Secretary of the General Assembly

Federico CASTELLUCCI

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)

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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)

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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

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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		(X-MW)^2
standard deviation (nkat/g)	1195.60		1203896.6
standard deviation %	15.77		5333533.6
Variance	248.5		30819.8
$s^2(r)$	1191221.0		168555.9
s(r)	1091.4		208.6
r (nkat/g) repeatability	3088.7	sum	410311.4
			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; 5 min			
mean value (nkat/g)	3896.4		(X-MW)^2
standard deviation (nkat/g)	367.08		95927.9
			94898.2

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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

standard deviation %	9.42		103290.8
Variance	88.76		36205.6
$s^2(r)$	112292.05		38787.1
s(r)	335.10		304642.7
r (nkat/g) repeatability	948.33	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			(X-MW)^2
mean value (nkat/g)	10445.83		49506.3
standard deviation (nkat/g)	445.29		400056.3
standard deviation %	4.26		20306.3
Variance	18.2		28617.4
$s^2(r)$	165237.7		447784.0
s(r)	406.5		45156.3
r (nkat/g) repeatability	1150.4	sum	991426.4

Enzyme	**Absorbance 5 min**	**Concentration (mg/ml)**	**U/g**	**nkat/g**

Enzyme 4; 5 min			(X-MW)^2

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Enzyme 4	0.2032	0.01	530.4	8840	mean value (nkat/g)	8751.7			7802.8
Enzyme 4	0.19614	0.01	505.5	8425	standard deviation (nkat/g)	420.38			106711.1
Enzyme 4	0.21	0.01	555.9	9265	standard deviation %	4.80			263511.1
Enzyme 4	0.19188	0.01	490.5	8175	Variance	23.1			332544.4
Enzyme 4	0.20858	0.01	549.3	9155	$s^2(r)$	147263.9			162677.8
Enzyme 4	0.3448	0.01	519	8650	s(r)	383.7			10336.1
					r (nkat/g) repeatability	1086.0		sum	883583.3

Enzyme	**Absorbance 5 min**	**Concentration (mg/ml)**	**U/g**	**nkat/g**	Enzyme 5; 5 min			(X-MW)^2
Enzyme 5	0.35063	0.01	978.1	16302	mean value (nkat/g)	16894.4		351385.5
Enzyme 5	0.35329	0.01	987.5	16458	standard deviation (nkat/g)	631.40		190192.9
Enzyme 5	0.3812	0.01	1085.7	18095	standard deviation %	3.74		1441333.6
Enzyme 5	0.35979	0.01	1010.4	16840	Variance	14.0		2964.2

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Enzyme 5	0.35941	0.01	1009.1	16818
Enzyme 5	0.4559	0.01	1011.2	16853

$s^2(r)$	332226.5		5792.9
s(r)	576.4		1690.1
r (nkat/g) repeatability	1631.2	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			(X-MW)^2
mean value (nkat/g)	16153.1		1808277.9
standard deviation (nkat/g)	908.69		513213.0
standard deviation %	5.63		483411.2
Variance	31.6		893550.1
$s^2(r)$	688095.8		41231.6
s(r)	829.5		388890.8
r (nkat/g) repeatability	2347.5	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; 10 min			(X-MW)^2
mean value (nkat/g)	8532.5		54056.3
standard deviation (nkat/g)	246.48		158006.3
standard deviation %	2.89		76084.0

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Enzyme 4	0.3420	0.01	509.5	8492	Variance	8.3			1667.4
Enzyme 4	0.3472	0.01	518.6	8643	$s^2(r)$	50627.5			12284.0
Enzyme 4	0.3448	0.01	514.4	8573	s(r)	225.0			1667.4
					r (nkat/g) repeatability	636.8		sum	303765.3

Enzyme	Absorbance 10 min	Concentration (mg/ml)	U/g	nkat/g	Enzyme 5; 10 min			(X-MW)^2
Enzyme 5	0.43542	0.01	638.3	10638	mean value (nkat/g)	11608.9		941978.1
Enzyme 5	0.49384	0.01	741.2	12353	standard deviation (nkat/g)	656.31		554197.5
Enzyme 5	0.4712	0.01	701.4	11690	standard deviation %	5.65		6579.0
Enzyme 5	0.49213	0.01	738.2	12303	Variance	32.0		482253.1
Enzyme 5	0.46232	0.01	685.7	11428	$s^2(r)$	358947.8		32600.3
Enzyme 5	0.4559	0.01	674.4	11240	s(r)	599.1		136079.0
					r (nkat/g) repeatability	1695.5	sum	2153687.0

Enzyme	Absorbance 10 min	Concentration (mg/ml)	U/g	nkat/g	Enzyme 6; 10 min			(X-MW)^2
Enzyme 6	0.60886	0.01	987.9	16465	mean value (nkat/g)	14436.1		4116390.1
Enzyme 6	0.5221	0.01	835.1	13918	standard deviation (nkat/g)	1012.31		268093.8

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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

standard deviation %	7.01		404637.3
Variance	49.2		228271.6
$s^2(r)$	853983.0		99926.2
s(r)	924.1		6579.0
r (nkat/g) repeatability	2615.2	sum	5123898.1

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

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