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.

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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.

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)

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- Vortex mixer

3. Chemicals and reagents

- Polygalacturonic acid, ~95 % enzymatic (CAS 25990-10-7)
- pH 4.0 Na-citrate/HCl buffer, 1.06 g/cm3 (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 H2O

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

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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.

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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

1. 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	**Absrobance 5 min**	**Concentration (mg/ml)**	**U/g**	**Nkat/g**	**Enzyme 1; 5 min**		(X-MW)^2
Enzyme 1	0.1698	0.01	389.2	6487	mean value (nkat/g)	7583.9	1203896.6
Enzyme 1	0.2278	0.01	593.6	9893	standard deviation (nkat/g)	1195.60	5333533.6
Enzyme 1	0.1855	0.01	444.5	7408	standard deviation %	15.77	30819.8
Enzyme 1	0.1815	0.01	430.4	7173	Variance	248.5	168555.9
Enzyme 1	0.1887	0.01	455.9	7598	$s^2(r)$	1191221.0	208.6
Enzyme 1	0.1776	0.01	416.6	6943	s(r)	1091.4	410311.4
					r (nkat/g) repeatability	3088.7	Sum= 7147325.9
					Enzyme 2; 5 min		(X-MW)^2
Enzyme 2	0.0898	0.01	215.2	3587	mean value (nkat/g)	3896.4	95927.9
Enzyme 2	0.0898	0.01	215.3	3588	standard deviation (nkat/g)	367.08	94898.2

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Enzyme 2	0.0897	0.01	214.5	3575	standard deviation %	9.42	103290.8
Enzyme 2	0.09	0.01	245.2	4087	Variance	88.76	36205.6
Enzyme 2	0.0954	0.01	245.6	4093	$s^2(r)$	112292.05	38787.1
Enzyme 2	0.0971	0.01	266.9	4448	s(r)	335.10	304642.7
					r (nkat/g) repeatability	948.33	Sum= 673752.3
					Enzyme 3; 5 min		(X-MW)^2
Enzyme 3	0.4077	0.01	613.4	10223	mean value (nkat/g)	10445.83	49506.3
Enzyme 3	0.3937	0.01	588.8	9813	standard deviation (nkat/g)	445.29	400056.3
Enzyme 3	0.4201	0.01	635.3	10588	standard deviation %	4.26	20306.3
Enzyme 3	0.4095	0.01	616.6	10277	Variance	18.2	28617.4
Enzyme 3	0.4381	0.01	666.9	11115	$s^2(r)$	165237.7	447784.0
Enzyme 3	0.4225	0.01	639.5	10658	s(r)	406.5	45156.3
					r (nkat/g) repeatability	1150.4	Sum= 991426.4
					Enzyme 4; 5 min		(X-MW)^2
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 5; 5 min		(X-MW)^2

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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
Enzyme 5	0.35941	0.01	1009.1	16818	$s^2(r)$	332226.5	5792.9
Enzyme 5	0.4559	0.01	1011.2	16853	s(r)	576.4	1690.1
					r (nkat/g) repeatability	1631.2	Sum= 1993359.3
					Enzyme 6; 5 min		(X-MW)^2
Enzyme 6	0.30006	0.01	888.5	14808	mean value (nkat/g)	16153.1	1808277.9
Enzyme 6	0.3108	0.01	926.2	15437	standard deviation (nkat/g)	908.69	513213.0
Enzyme 6	0.3348	0.01	1010.9	16848	standard deviation %	5.63	483411.2
Enzyme 6	0.3391	0.01	1025.9	17098	Variance	31.6	893550.1
Enzyme 6	0.3195	0.01	957	15950	$s^2(r)$	688095.8	41231.6
Enzyme 6	0.5370	0.01	1006.6	16777	s(r)	829.5	388890.8
					r (nkat/g) repeatability	2347.5	Sum: 4128574.5
					Enzyme 4; 10 min		(X-MW)^2
Enzyme 4	0.3355	0.01	498	8300	mean value (nkat/g)	8532.5	54056.3
Enzyme 4	0.3569	0.01	535.8	8930	standard deviation (nkat/g)	246.48	158006.3
Enzyme 4	0.3340	0.01	495.4	8257	standard deviation %	2.89	76084.0
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

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					r (nkat/g) repeatability	636.8	Sum: 303765.3
					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 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
Enzyme 6	0.5180	0.01	828.0	13800	standard deviation %	7.01	404637.3
Enzyme 6	0.52344	0.01	837.5	13958	Variance	49.2	228271.6
Enzyme 6	0.52895	0.01	847.2	14120	$s^2(r)$	853983.0	99926.2
Enzyme 6	0.537	0.01	861.3	14355	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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