

SOP for the Determination of Galacturonic Content

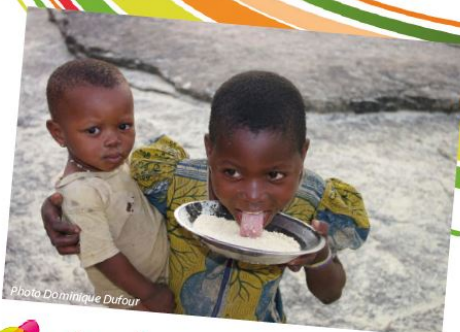
Biophysical Characterization of Quality Traits, WP2

Montpellier, France, November 2021

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This SOP has been written in the framework of RTBfoods project.


To be cited as:

Christian MESTRES, Lea OLLIER, Romain DOMINGO (2022). *SOP for Determination of Galacturonic Content. Biophysical Characterization of Quality Traits, WP2.* Montpellier, France: RTBfoods Laboratory Standard Operating Procedure, 12 p.

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Acknowledgments: This work was supported by the RTBfoods project <https://rtbfoods.cirad.fr>, through a grant OPP1178942: Breeding RTB products for end user preferences (RTBfoods), to the French Agricultural Research Centre for International Development (CIRAD), Montpellier, France, by the Bill & Melinda Gates Foundation (BMGF).

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<p align="center">RTBfoods</p> <p>WP2: Biophysical Characterization of Quality Traits</p>		
<p align="center">SOP: Determination of the galacturonic acid content</p>		
<p align="center">Date: 30/12/2021</p>	<p align="center">Release: 1</p>	
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ABSTRACT

Cell walls in plants are mainly composed of polysaccharides. Among them, pectin, cellulose and hemicellulose are composed of various sugars residues (acidic: galacturonic acid in pectins; or neutral: rhamnose, galactose, arabinose, xylose, glucose in cellulose, hemicellulose and pectic side chain). This fine composition affects the structure but also the behavior and mechanical properties of cell walls and consequently the textural properties of RTB foods. In raw products and/or after cooking some cell wall components are soluble (eg. some pectin).

This procedure seeks to determine the galacturonic acid level extracted from pre-dried (dried or freeze-dried) RTBs. This method gives the galacturonic acid content of cell walls extracted with different solvents, independently of the neutral sugars content.

Pectin are first extracted at intermediate temperature (55°C) to facilitate their extraction while limiting their chemical and enzymatic degradation and the extraction of starch (temperature below starch gelatinization). Extracted pectin are then hydrolyzed by hot sulphuric acid in order to obtain sugar residues and their furfural derivatives. Neutral sugars gave brown derivatives whereas free galacturonic acid is quantified by colorimetry after reaction with MHDP which produces a specific fuschia pink complex with a maximum absorption at 520 nm (Blumenkrantz and Asboe-Hansen, 1973). Galacturonic acid content is calculated after subtracting the brown colour (at 520 nm) generated by neutral sugar hydrolysis.

Key Words: pectin, galacturonic acid, extraction, colorimetric measurement

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1 SCOPE AND APPLICATION

Cell walls in plants are mainly composed of polysaccharides. Among them, pectin, cellulose and hemicellulose are composed of various sugars residues (acidic: galacturonic acid in pectins; or neutral: rhamnose, galactose, arabinose, xylose, glucose in cellulose, hemicellulose and pectic side chain). This fine composition affects the structure but also the behavior and mechanical properties of cell walls and consequently the textural properties of RTB foods. In raw products and/or after cooking some cell wall components are soluble (eg. some pectin).

This procedure seeks to determine the pectin level extracted from pre-dried (dried or freeze-dried) RTBs. This method gives the galacturonic acid content of cell walls extracted with different solvents, independently of the neutral sugars content.

2 REFERENCES

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry*, 54, 484–489.

Goldberg R., Morvan C., Jauneau A. & Jarvis M.C. (1996) Methylesterification, de-esterification and gelation of pectins in the primary cell wall. In *Pectins and Pectinases* (eds J. Visser & A.G.J. Voragen), pp. 561–568. Elsevier, Amsterdam, The Netherlands.

3 DEFINITIONS

Acidic sugars are generally monosaccharides comprising a carboxyl function: galacturonic acid is the main acidic sugar in plants.

Neutral sugars are mono- or di-saccharides with only ketone or aldehyde function as carbonyl group.

Pectin are polysaccharides composed of a backbone of anhydro- α -D-galacturonic acid and small amounts of α -L-rhamnose on which neutral sugars are branched. α -D-galacturonic acid generally represents by 50% of pectin composition. One part of the acidic groups of the pectin backbone are esterified, mainly by methanol.

EDTA: Ethylene-diamine-tetra-acetic acid is a chelator of divalent cations.

MHDP (m-hydroxydiphenyl) or 3-phenylphenol is a specific reagent of uronic acids.

4 PRINCIPLE

Pectin are first extracted at intermediate temperature (55°C) to facilitate their extraction while limiting their chemical and enzymatic degradation and the extraction of starch (temperature below starch gelatinization). Three type of solvents can be used:

- pH 6.5 buffer + EDTA to get the highly soluble pectin, that are not linked by ester or labile amide linkages
- pH 10 to extract highly methylated pectin. Mild alkaline extraction cleaves ester or labile amide linkages, and then solubilizes a substantial pectic fraction (Goldberg et al. 1996). Poorly methylated pectin remains however insoluble due to interaction with divalent cations,
- pH 10 + EDTA to extract most pectin.

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Extracted pectin are then hydrolyzed by hot sulphuric acid in order to obtain sugar residues and their furfural derivatives. Neutral sugars gave brown derivatives whereas free galacturonic acid is quantified by colorimetry after reaction with MHDP which produces a specific fuschia pink complex with a maximum absorption at 520 nm (Blumenkrantz and Asboe-Hansen, 1973). Galacturonic acid content is calculated after subtracting the brown colour (at 520 nm) generated by neutral sugar hydrolysis.

5 REAGENTS & SOLUTIONS

5.1 Reagents

All commercially obtained chemical reagents are listed in Table 1 and are used without further purification.

Table 1. Commercially obtained chemicals

Reagent	CAS number	Supplier (example)	Product number (example)
Sulfuric acid (95-97%)	7664-93-9	Merck	84727
3-phenylphenol (≥ 85%)	580-51-8	Merck	262250-5G
EDTA (≥ 98.5%)	60-00-4	Merck	ED-100G
Sodium hydroxide (≥ 97%)	1310-73-2	Merck	221465-500 G
Sodium carbonate monohydrate (≥ 99.5%)	5968-11-6	Merck	230952-100G
Sodium bicarbonate (≥ 99.7%)	144-55-8	Merck	S6014-500G
D-(+)-Galacturonic acid monohydrate (> 97%)	685-73-4	Merck	48280

5.2 Solutions

- **4M NaOH:** 40 g of Sodium hydroxide pellet (1310-73-2) in 250 mL ultrapure or distilled water
- **125mM NaOH:** 2.5 g of Sodium hydroxide pellet (1310-73-2) in 500 mL ultrapure or distilled water
- **0.15% (m/V) 3-phenylphenol (580-51-8):** 150 mg of 3-phenylphenol in 100 mL of 125 mM NaOH
- **pH 6.5 + EDTA 0.05 M buffer:** Dissolve 14.612 g of EDTA (60-00-4) in 200 mL of ultrapure water. Add concentrated 4M NaOH solution to reach a pH of 6.5 and make up to 1 L with ultrapure or distilled water.
- **pH 10 buffer (50 mM):**
 - Solution A: 3.1 g of sodium carbonate monohydrate (Na_2CO_3 , 5968-11-6) in 500 mL of ultrapure water

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- Solution B: 2.1 g of sodium bicarbonate ($\text{NaHCO}_3 \cdot \text{H}_2\text{O}$, 144-55-8) in 500 mL of ultrapure water
- Measure 275 mL of solution A and gradually add solution B (≈ 225 mL) until the pH reaches 10.
- **pH 10 + EDTA buffer:** Dissolve 14.612 g of EDTA (60-00-4) in 200 mL of ultrapure water. Add concentrated 4M NaOH solution to reach a pH of 10 and make up to 1 L with ultrapure distilled water.
- **Standard solution:**
 - prepare a stock solution of galacturonic acid (685-73-4) at 1 mg/ml (taking into account the hydration level and purity of the reagent: for example, weigh 22.6 mg of 48280 Merck reagent to get 20 mg of galacturonic acid ($20/0.97 \cdot 212/194$) that is solubilized in 20 mL of ultrapure or distilled water
 - then dilute to 50 $\mu\text{g}/\text{ml}$ in ultrapure or distilled water

6 APPARATUS

- High precision (0.01 mg) balance
- Shaking water bath (55°C)
- Tube vortex stirrer
- pH meter
- 50 mL screw centrifuge tubes (hermetically closing) (for example Falcon tubes)
- 20 mL test tubes
- Spectrophotometer

7 PROCEDURE

7.1 Extraction

- Turn on the water bath at 55 °C (at least 1 hour before extraction)
- In a 50 mL screw centrifuge tube, weigh out approximately 250 mg of previously ground sample.
- Add 10 mL of extraction buffer (pH 6.5+EDTA, pH 10 or pH10+EDTA), and disperse with a vortex.
- Place the tubes in a water bath lying in the tulips with gentle back and forth agitation for one hour.
- After 1 hour, centrifuge at room temperature, 4000 rpm, 10 min
- Use the supernatant directly for galacturonic acid measurement or store at 4°C for a maximum of 24 hours.

7.2 Dilution, hydrolysis and colorimetric measurement

7.2.1 Dilutions

Each supernatant of the extracted pectin is diluted with ultrapure water, generally between 5 and 10 times, to get an OD (Optical Density or Absorbance) in the range of the standard solutions (if the OD

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measured for the sample is over the maximum value of the standard solutions, the dilution is increased and the hydrolysis and colorimetric reaction performed again).

For the standard solutions, place the exact volumes of diluted galacturonic acid solution and ultrapure water in each 20 mL test tube as described below (table 2):

Final galacturonic acid concentration (µg/mL)	Volume (µl) of diluted galacturonic acid solution (50µg/ml)	Volume of ultrapure water (µl)
0	0	400
12.5	100	300
25	200	200
37.5	300	100
50	400	0

Table 2: Calibration solutions

7.2.2 Hydrolysis and colorimetric measurement

In a 20 mL test tube:

- place 400 µL of the diluted supernatant of the pectin extract or of the standard solutions, and place the tube in a cold bath (water + ice cubes)
- add 2.4 ml of concentrated (95-97%) sulphuric acid, shake very strongly for few seconds with a Vortex stirrer and quickly return to the cold bath (water + ice cubes)
- place in a boiling water bath for exactly 10 minutes (shake with a Vortex stirrer after 5 & 9 minutes)
- cool in an ice bath,
- add 40µl of 0.15% 3-phenylphenol solution (galacturonic acid measurement) **or** 40µl of 125mM NaOH (sugar bias measurement)
- Stirr well, then immediately place in a water bath at 35°C for exactly 15 minutes, then take the OD reading at 520 nm; each tube should be separated by 30s.

8 EXPRESSION OF RESULTS

8.1 Method of calculation and formulae

8.1.1 Calibration

A calibration curve is performed each day (see figure 1). The determination coefficient is normally very close to one. The slope (SL) of the regression is used to calculate the galacturonic acid level.

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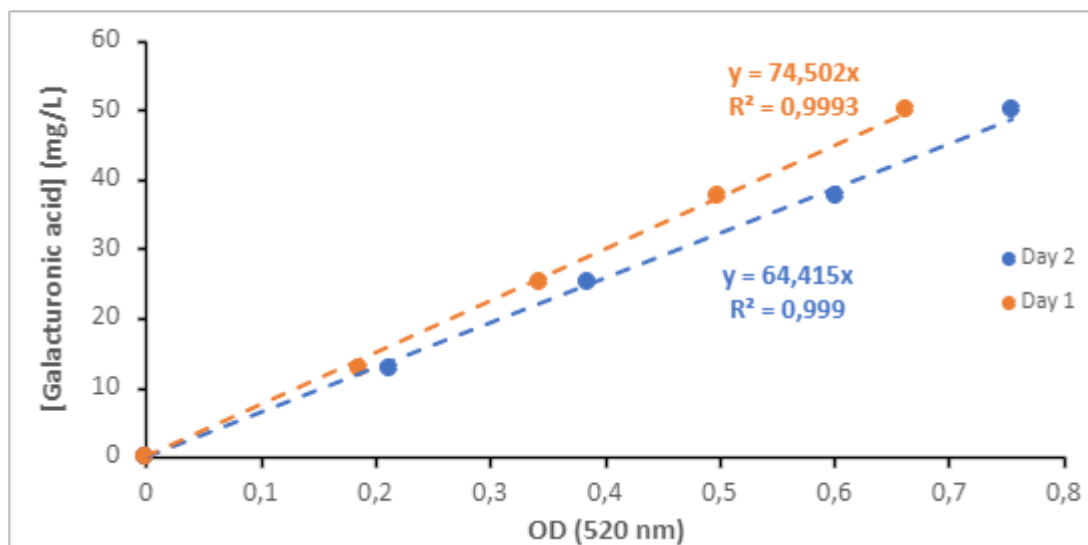


Figure 1: Calibration curve examples

8.1.2 Formulae

The galacturonic acid content (g/100 g) is calculated according to the following equation:

$$[\text{Galacturonic acid}] = \frac{OD_{520} \times SL \times VE \times FD \times 100}{\text{Mass} \times 1000}$$

Where:

OD_{520} is the difference between the OD measured at 520 nm with 3-phenylphenol minus the OD measured with 125mM NaOH

SL is the slope of the calibration curve

VE is the volume of extraction (10 mL)

FD is the factor of dilution

Mass is the mass of sample (expressed in mg) submitted to pectin extraction

1000 is for the conversion of L to mL

100 to express the results as %

8.2 Repeatability

The coefficient of variation of the Galacturonic acid content between measurements performed in a short time interval will be less than 10% (see Table 3).

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Sample	Replication	Mass (mg)	VE (mL)	FD	SL	OD ₅₂₀ with 3-phenylphenol	OD ₅₂₀ with 125mM NaOH	[Gal acid] mg/L	[Gal acid] (g/100g)	Mean value [Gal acid] (g/100g)	Coefficient of variation (% mean value)
Florido tub 1 centr	1	246.8	10	5	74.5	0.505	0.07	162	0.66	0.64	6.4
	2	246.8	10	5	65.3	0.744	0.24	164.6	0.67		
	3	246.8	10	5	64.4	0.516	0.063	145.9	0.59		
H4X172 tub 1 prox	1	243.9	10	10	74.5	0.603	0.113	365.1	1.5	1.49	5.7
	2	243.9	10	10	65.3	0.727	0.138	384.6	1.58		
	3	243.9	10	10	64.4	0.647	0.114	343.3	1.41		
Kinabayo tub 3 prox	1	253.1	10	5	74.5	0.675	0.13	203	0.8	0.73	8.9
	2	253.1	10	5	65.3	0.671	0.11	183.2	0.72		
	3	253.1	10	5	64.4	0.634	0.105	170.3	0.67		
Plimbite tub 1 centr	1	242.1	10	5	74.5	0.424	0.05	139.3	0.58	0.57	6.1
	2	242.1	10	5	65.3	0.496	0.055	144	0.59		
	3	242.1	10	5	64.4	0.435	0.038	127.8	0.53		

Table 3: Example of results

9 CRITICAL POINTS OR NOTE ON THE PROCEDURE

Critical points:

- An assay should be limited to 30 tubes in order to respect a reaction time of 15 minutes in the 3-phenylphenol reaction step, if each tube is offset from the previous one by 30s.
- For the hydrolysis phase, the 30 tubes are prepared and mixed cold together, placed together in the boiling water bath, then placed together in the ice bath.
- Intense stirring is very important after addition of sulphuric acid as phase separation occurs
- Mix coloured complex very well to get a reproducible absorbance; at least after adding 3-phenylphenol, after 10 minutes of incubation and just before reading, as phase separation tends to occur.

Notes:

- The solution of 3-phenylphenol can only be stored for one week
- Caution: Concentrated sulphuric acid is very corrosive

10 TEST REPORT

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification, preparation of the sample and calculation (see Table 3 as example).



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