Laboratory Standard Operating Procedure

Sample Preparation for Cell Wall Polysaccharides Analysis of Raw and Boiled Yam and Plantain

Biophysical Characterization of Quality Traits, WP2

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Agnès ROLLAND-SABATÉ, Institut National de Recherche pour l'Agriculture, l'alimentation et l'environnement (INRAe), Avignon, France

Aliénor DUTHEIL DE LA ROCHÈRE, INRAe, Avignon, France

Mark TAYLOR, James Hutton Institute, (JHI), Dundee, UK (Validator)

Christian MESTRES, Centre de coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Montpellier, France (Validator)

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Ethics: The activities, which led to the production of this manual, were assessed and approved by the CIRAD Ethics Committee (H2020 ethics self-assessment procedure). When relevant, samples were prepared according to good hygiene and manufacturing practices. When external participants were involved in an activity, they were priorly informed about the objective of the activity and explained that their participation was entirely voluntary, that they could stop the interview at any point and that their responses would be anonymous and securely stored by the research team for research purposes. Written consent (signature) was systematically sought from sensory panelists and from consumers participating in activities.

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Date: 22/03/2021	**Release: 1**
Written by: Aliénor DUTHEIL DE LA ROCHÈRE, Agnès ROLLAND-SABATÉ For information on this SOP please contact: • Aliénor DUTHEIL DE LA ROCHÈRE, alienor.dutheil-de-la-rochere@inrae.fr • Agnès ROLLAND-SABATÉ, agnes.rolland-sabate@inrae.fr	
This document has been reviewed by:	
Mark TAYLOR (JHI) Christian MESTRES (CIRAD)	
Final validation by:	
Christian MESTRES	13/01/2022

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ABSTRACT

This method seeks to describe the preparation of samples for cell wall polysaccharides analysis in order to assess the amount of the different types of sugars contained in the cell walls of raw and boiled yam and plantain. The extraction of cell wall polysaccharides (CWP) involves several steps. Yam and plantain are rich in starch polysaccharides (>80% d.b.) in order to obtain reliable and measurable data on cell wall polysaccharides, starch removal is mandatory before cell wall analysis. This step has been particularly optimized. This SOP has been developed on commercial yam and green plantain, as they are particularly high in starch. It was then applied to various yam and banana samples at different ripening stages (fresh fruits, cold powders or freeze-dried). The preparation of CWP samples from amylaceous products requires 4 steps: (i) extraction of alcohol insoluble solids (AIS) to remove all the soluble sugars and polyphenols, (ii) starch removal in aqueous solution thanks to enzymatic reactions, (iii) quenching by 96% EtOH, followed by new extraction of destarched AIS, to remove the soluble sugars and polypeptides produced by the starch removal process, and finally (iv) starch assay to assess the efficiency of the starch removal process.

Key Words (10 maximum): cell wall preparation for analysis, polysaccharides of raw and boiled yam, polysaccharides of raw and boiled plantain

1 SCOPE AND APPLICATION

This SOP describes the preparation of samples for cell wall polysaccharides analysis in order to assess the amount of the different types of sugars contained in the cell walls. The extraction of cell wall polysaccharides (CWP) has several steps, amongst which starch removal is the hardest. This SOP has been developed on commercial yam and green plantain, as they are high in starch.

2 REFERENCES

The SOP relies on the following references:

Shiga, Tânia M., Nicholas C. Carpita, Franco Maria Lajolo, et Beatriz Rosana Cordenunsi-Lysenko. 2017. « Two Banana Cultivars Differ in Composition of Potentially Immunomodulatory Mannan and Arabinogalactan ». *Carbohydrate Polymers* 164 (mai): 31-41. https://doi.org/10.1016/j.carbpol.2017.01.079

Ngolong Ngea, Guillaume Legrand, Fabienne Guillon, Jean Justin Essia Ngang, Estelle Bonnin, Brigitte Bouchet, et Luc Saulnier. 2016. « Modification of Cell Wall Polysaccharides during Retting of Cassava Roots ». *Food Chemistry* 213 (décembre): 402-9. https://doi.org/10.1016/j.foodchem.2016.06.107.

3 DEFINITIONS

CWP: cell wall polysaccharides

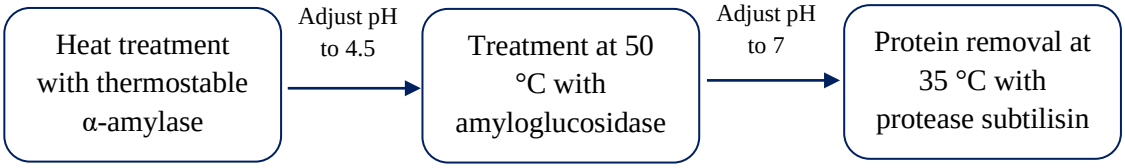
Alcohol Insoluble Solids (AIS): Fraction of the plant sample that is insoluble in alcohol. AIS is mainly composed of polysaccharides (starch and CWP).

Subtilisin: Protease, enzyme which catalyses the breakdown of proteins (such as enzymes) into smaller and soluble polypeptides.

4 PRINCIPLE

The preparation of CWP samples from amylaceous products is carried out on fresh fruits, cold powders or freeze-dried powders. It requires 4 steps:

1. Extraction of AIS which removes all the soluble sugars and polyphenols
2. Starch removal, in aqueous solution:



3. Quenching by 96% EtOH, followed by new extraction of destarched AIS, to remove the soluble sugars and polypeptides produced by the starch removal process
4. Starch assay to assess the efficiency of the starch removal process

5 REAGENTS

Reagent	Purity or concentration	CAS	Brand
Ethanol	96% and 70% aqueous solutions	64-17-5	VWR
Acetone	60% and 80% aqueous solutions; 100%	67-64-1	Carlo Erba
Sulfuric acid	95-98%	7664-93-9	VWR International, Leuven, Belgium
Phenol	50g/L aqueous solution	108-95-2	Sigma-Aldrich, Steinheim, Germany
Thermostable α-amylase	3000 U/mL; 54 U/mg	9000-90-2, 9000-85-5	Megazyme
Amyloglucosidase	3260 U/mL	9032-08-0	Megazyme
Subtilisin A	50 mg/mL; 6 U/mg	9014-01-1	Megazyme
Starch Assay Kit			Megazyme
Acetate buffer solution	pH 4.5		
Sodium hydroxide	1M aqueous solution	1310-73-2	VWR International, Leuven, Belgium

6 APPARATUS

a. Heat block (for the starch assay)
b. UV-vis spectrophotometer (for the starch assay)
c. 3 Shaking water baths (or 2 shaking water baths and a static water bath)
d. Laboratory oven
e. Laboratory centrifuge
f. Magnetic stirrer
g. Sep-pack columns extractor
h. pHmeter (or pH strips)

7 PROCEDURE

7.1 Extraction of Alcohol Insoluble Solids (AIS)

Extraction of AIS can be made using either Sep-pack column extractor with 20-µm sinter or centrifuge. Column extractor is not recommended for fresh yam nor cold yam powder. The first steps (7.1.1 or 7.1.2 regarding the type of powder) are the same regardless the type of extraction chosen.

7.1.1 First extraction step for cold powders or fresh pulp

5. Weigh 16 g of pulp of cold powder in a 400 mL beaker with a magnetic stirrer
6. Add 80 mL boiling EtOH 96%
7. Stir while keeping it at boiling temperature on a heating stirring device for 10 minutes
8. Stir it overnight at 4 °C

7.1.2 First extraction step for freeze-dried powders

1. Weigh 5 g of freeze-dried powder in a 150 mL beaker with a magnetic stirrer
2. Add 250 mL boiling EtOH 70%
3. Stir while keeping it at boiling temperature on a heating stirring device for 10 minutes
4. Stir it overnight at 4 °C

7.1.3 Extraction process on Sep-pack column extractor

1. Place 2 to 3 weight 60 mL columns with 20 µm sinters on the vacuum extractor.
2. Using 700 mL EtOH 70% and the extractor, rinse the suspension obtained during the first step.
3. Assess the absence of remaining soluble sugars with a colorimetric assay: in a test tube, add 0.5 mL of permeate to 0.5 mL of phenol solution and 3 mL of sulfuric acid. If the solution turns yellow, the retentate has to be rinsed again with 70% EtOH.
4. Rinse the retentate with 150 mL 60% Acetone, then 150 mL 80% Acetone, then 300 mL 100% acetone.
5. Dry in a laboratory oven at 35 °C.
6. Weigh the dried columns to assess the AIS extraction yield.

7.1.4 Extraction process via centrifugation

1. Weigh six 50 mL Falcon tubes.
2. Fill each Falcon tube with 42.5 mL of the suspension obtained during the first step, completed if needed with EtOH 70%. Close and shake vigorously each tube.
3. Balance the tubes within a 0.05 g range by addition of EtOH 70% and centrifuge for 7 minutes at 9000 rpm and 15 °C.
4. Throw away the supernatant, add 70% EtOH and repeat the process 4 times.
5. Assess the absence of remaining soluble sugars with a colorimetric assay: in a test tube, add 0.5 mL of supernatant to 0.5 mL of phenol solution and 3 mL of sulfuric acid. If the solution turns yellow, the retentate has to be rinsed again two times with 70% EtOH.

6. Repeat the extraction process once with 60% Acetone, then once with 80% Acetone, then twice with 100% acetone.
7. Dry in a laboratory oven at 35 °C.
8. Weigh the dried Falcon tubes to assess the AIS extraction yield.

7.1.5 Starch removal on yam AIS

1- *Starch removal is carried out on AIS. It shall not be carried out on plant powders directly, as polyphenols inhibit the activity of one of the enzymes used in the starch removal process.* Weigh 8 g of AIS in a 400 mL beaker

2- Add 200 mL of deionized water and stir for one hour at room temperature.

3- In the meantime, heat three shaking water baths, one up to 95 °C, the second to 75 °C and the last to 50 °C. Turn on the laboratory oven to 35 °C.

4- After stirring the AIS suspension for one hour, add 3.2 mL of thermostable α-amylase, stir for 10 seconds and put at 95 °C for 12 minutes (if the water bath does not have a shaking feature, stir magnetically after 12 minutes).

5- Transfer to a 75°C shaking water bath for 30 minutes.

6- Remove the beaker from the water bath. Add 15 mL of buffered solution at pH 4.5.

7- Heat at 50 °C for 10 minutes.

8- Add 1.92 mL of amyloglucosidase, stir manually and heat at 50 °C for 30 minutes.

9- Remove from the 50 °C water bath. Adjust the pH to 7 by addition of NaOH 1M (approx. 1.8 mL).

10- Add 3.2 mL Subtilisin A and put in the laboratory oven at 35 °C with a magnetic stirring device for one hour.

11- Using a 1000 mL beaker to transfer the suspension into, quench the process by adding 480 mL of 96% EtOH (final total volume of the suspension: 680 mL).

12- Stir overnight at 4 °C.

7.1.6 Starch removal on plantain AIS

1- *Starch removal is carried out on AIS. It shall not be carried out on plant powders directly, as polyphenols inhibit the activity of one of the enzymes used in the starch removal process.* Weigh 4 g of AIS in a 400 mL beaker

2- Add 200 mL of deionized water and stir for one hour at room temperature.

3- In the meantime, heat three shaking water baths, one up to 95 °C, the second to 70 °C and the last to 50 °C. Turn on the laboratory oven to 35 °C.

4- After stirring the AIS suspension for one hour, add 1.6 mL of thermostable α-amylase, stir for 10 seconds and put at 95 °C for 12 minutes (if the water bath does not have a shaking feature, stir magnetically after 12 minutes).

5- Transfer to a 70°C shaking water bath for 20 minutes.

6- Remove the beaker from the water bath. Add 15 mL of buffered solution at pH 4.5.

7- Heat at 50 °C for 10 minutes.

8- Add 0.96 mL of amyloglucosidase, stir manually and heat at 50 °C for 30 minutes.

9- Remove from the 50 °C water bath. Adjust the pH to 7 by addition of NaOH 1M (approx. 1.8 mL).

10- Add 1.6 mL Subtilisin A and put in the laboratory oven at 35 °C with a magnetic stirring device for one hour.

11- Using a 1000 mL beaker to transfer the suspension into, quench the process by adding 480 mL of 96% EtOH (final total volume of the suspension: 680 mL).

12- Stir overnight at 4 °C.

7.1.7 Extraction of destarched AIS

This step follows the process described in 7.1.4. Once all the 680 mL suspension (containing 70% EtOH) has been centrifuged, rinse and centrifuge 3 to 4 times with 70% EtOH before testing the supernatant with phenol.

7.1.8 Starch assay on destarched AIS

Starch concentration is assessed using the "Starch assay kit" from Megazyme. The protocol for non-resistant starch content is applied.

8 RESULTS

8.1.1 Global and step by step yield

To assess the yield and therefore the CWP content of the fresh fruit, one has to assess each step's yield; including the drying process for dried or freeze-dried powders (i.e. water content shall be known). It is approximated that freeze-dried and dried powders have no water at all to assess the different yields.

For the AIS extraction carried out on cold powders or fresh fruits, the yield on dry basis is calculated as follows: $\mathbf{Y_{AIS}(\%) = 100*m_{AIS}/[m_{initial}*(100-WaterContent\%)/100)]}$

For the AIS extraction carried out on dried or freeze-dried powders, the yield is calculated as follows: $\mathbf{Y_{AIS}(\%) = 100*m_{AIS}/m_{initial}}$

If water content is unknown, a yield on dry matter can be assessed with the following formula: $\mathbf{Y_{AIS\_Dry}(\%) = 100*m_{AIS}/m_{initial\_powder}}$

For the starch removal step, the yield is calculated as follows: $\mathbf{Y_{Destarch}(\%) = 100*m_{Destarched}/m_{AIS\_initial}}$ where $m_{AIS\_initial}$ is the mass of AIS weighed at the beginning of the destarching process.

The global yield of the whole process which assesses the percentage of CWP in fresh fruits is then: $\mathbf{Y_{CWP}(\%) = Y_{AIS}* Y_{Destarch}/100}$

The global yield on dry matter can also be calculated as follows: $\mathbf{Y_{CWP\_Dry}(\%) = Y_{AIS\_Dry}* Y_{Destarch}/100}$

Institute: Cirad – UMR QualiSud

Address: C/O Cathy Méjean, TA-B95/15 - 73 rue Jean-François Breton - 34398 Montpellier Cedex 5 - France

Tel: +33 4 67 61 44 31

Email: rtbfoodspmu@cirad.fr

Website: https://rtbfoods.cirad.fr/