Laboratory Standard Operating Procedure

SOP for Extraction, Quantification and Analysis of Phenol Acid Components of Cassava

Biophysical Characterization of Quality Traits, WP2

Montpellier, France, January 2023

Didier MBEGUIE-A-MBEGUIE, Centre de Recherche International en Agronomie pour le Développement (CIRAD), Montpellier, France

Noël AKISSOE, Université d'Abomey-Calavi, Faculté des Sciences Agronomiques (UAC-FSA), Cotonou, Benin (Validator)

Carine LE BOURVELLEC, Institut National de Recherche pour l'Agriculture, l'alimentation et l'environnement (INRAe), Avignon, France (Validator)

Agnès ROLLAND-SABATE, INRAe, Avignon, France (Validator)

Christian MESTRES, CIRAD, Montpellier, France (Validator)

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

To be cited as:

Didier MBEGUIE-A-MBEGUIE, Noël AKISSOE, Carine LE BOURVELLEC, Agnès ROLLAND-SABATE, Christian MESTRES (2023). *SOP for Extraction, Quantification and Analysis of Phenol Acid Components of Cassava. Biophysical Characterization of Quality Traits, WP2.* Montpellier, France: RTBfoods Laboratory Standard Operating Procedure, 15 p. https://doi.org/10.18167/agritrop/00744

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.

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).

RTBfoods *WP2: Biophysical Characterization of Quality Traits*	

SOP: Extraction, Quantification and Analysis of Phenol Acid Components of Cassava

Date: 20/01/2023	**Release: 1**

Written by: Didier MBEGUIE-A-MBEGUIE

For information on this SOP please contact:

- Didier MBEGUIE-A-MBEGUIE, didier.mbeguie-a-mbeguier@cirad.fr

This document has been reviewed by:

Noël AKISSOE (UAC-FSA)	17/12/2019
Carine LE BOURVELLEC (INRAe)	18/12/2019
Agnès ROLLAND-SABATE (INRAe)	18/12/2019

Final validation by:

Christian MESTRES (CIRAD)	20/01/2023

CONTENTS

Table of contents

ABSTRACT

Phenolic acids (PA) compounds are diverse, ubiquitous and widespread in nature. Thee biological active compound exhibit various activities including antimicrobial, antioxidant and anti-inflammatory. They can also be involved in physiological disorder mechanisms such as browning/discoloration of products that occur during processing and/or storage, thus reducing the product quality and its acceptability by consumers. Therefore, PA can be considered as putative biochemical indicators of some product quality traits associated with consumer preferences. We describe here a standard operating protocol (SOP) adapted from cassava leading to extract and quantify both free and bound PA compounds. It is based on a cold liquid-liquid extraction for extractable PA with an organic solvent methanol/water. Bound-PA are removed by cold alkaline or hot acid hydrolysis following by a solubilization into diethyl ether. The obtained PA were quantified using Folin Ciocalteu colorimetric method according to the standard protocol ISO 14502-1:2005 (E). This SOP was used to extract PA compounds from 6 contrasted cassava genotypes, harvested in two localities in Cameroon. The results shown that the PA content varied according to the genotype and the locality. The bound-PA form content was two-fold higher than the free form, the acid-hydrolysable form being also two-fold higher than the alkaline-hydrolysable form.

Keywords: Cassava, Phenolic Aacids Compounds, Quality, Retting abilityType of document

1 SCOPE AND APPLICATION

The present standard protocol adapted from wheat bran [1], describes the method of extraction of free and bound phenolic acid components from cassava using organic solvent, alkaline and acidic hydrolyses. It also describes the quantification of total polyphenol acids using standard ISO 14502-1:2005 protocol [2,3], and the HPLC-DAD conditions used to separate and quantify the different phenolic acid components.

2 REFERENCES

[1] Kim K.-H., Tsao R., Yang R. and Cui S. W. 2006. Phenolic acid profiles and antioxidants activities of wheat bran extracts and the effect of hydrolysis conditions. Food Chem. 95: 466-473.

[2] Singleton V. L., Orthofer R., Lamuela-Raventos R. M. 1999. Analysis of total phenolic and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. In: Methods in Enzymology. Oxidants and Antioxidants, Part A, Lester packer, ed. 299:152-178.

[3] ISO 14502-1:2005. Determination of substances characteristic of green and black tea — Part 1: Content of total polyphenols in tea — Colorimetric method using Folin-Ciocalteu reagent.

3 DEFINITIONS

FC: Folin-Ciocalteu reagent
MeOH: methanol
NaCl: sodium chloride
PA: phenolic acids
EGA: Equivalent Gallic acid
MeFPA: methanol-extractable free phenolic acids
AHPA: acid-hydrolysable phenolic acids
BHPA: alkaline-hydrolysable phenolic acids
bBHPA: bound alkaline-hydrolysable phenolic acids
bAHPA: bound acid-hydrolysable phenolic acids

4 PRINCIPLE

Plant PA compound are found as esters or glycosides or as free molecules, so their extraction method should be planned carefully as it affects the selectivity, yield and recovery of desired compound(s). They are found in soluble form (conjugated with sugars or organic acids) and bound with cell wall fractions. The method presented here was developed and adapted for a sample of 1 g of freeze-dried cassava powder and aim to extract both free and bound phenolic acid. It is based on a cold liquid-liquid extraction for extractable PA with an organic solvent methanol/water. Bound phenolic acids are removed by cold alkaline or hot acid hydrolysis following by a solubilization into diethyl ether.

Total phenolic acids are determined by FC colorimetric method according to the standard protocol ISO 14502-1:2005 (E). This method is based on a reduction of FC component (phosphotungstic acid (H3PW12O40) and phosphomolybdic acid (H3PMo12O40)) during oxidation of the phenolic compound leading to blue mixture of tungsten oxide (W8O23) and molybdene (Mo8O23). The blue

coloration produced has a maximum absorption between 620 et 760 nm. It is proportional to the level of phenolic compounds content.

The flowchart of the phenolic acid extraction and determination is described in appendix (Figure 1) and an example of results is given in Table 1.

5 REAGENTS

All reagents used must be analytical grade ones, unless otherwise specified.

- **Acetonitrile, for HPLC gradient grade**
- **Hydrochloric acid (HCl), concentrated (37%, ~12M)**
- **Diethyl ether**
- **Formic acid, for HPLC gradient grade**
- **Methanol, for HPLC gradient grade**
- **Water, ultrapure or distillated**

  Folin-Ciocalteu reagent used for calculation of total phenol content (Sigma, 47641)

  Gallic acid standard solutions (Sigma, 27645)

- **Acidic water**

  In a 100 mL one-mark volumetric flask (6.5), add 70 mL of ultrapure water (5.6), adjust the pH at 2 with a 37% HCl solution (5.2) and complete with distillated water to 100 mL.

- **Formic acid solution, 1% (volume fraction)**

  Using a pipette, transfer 20 mL of Formic acid pure to a 1 L one-mark volumetric flask (6.5). Dilute to the mark with Ultrapure Water (5.6) and mix.

  Note: the solution will remain stable at room temperature for up to 1 week.

- **Methanol/water extraction solvent, 80% (volume fraction)**

  Add 800 mL of methanol analytical grade in a 1 L one-mark volumetric flask (6.5). Dilute to the mark with Ultrapurewater (5.6) and mix.

  Note: the solution will remain stable at room temperature for up to 1 week.

- **Hydrochloric acid (HCl), 3 M solution**

  In a 100 mL one-mark volumetric flask (6.5), add 70 mLof distilled water (5.6). Add 25 mL of concentrated hydrochloric acid solution (5.2) and adjust with distilled water up to 100 mL.

  Note: Hydrochloric acid is a hazardous product that must be handled under a ventilated chemical hood. For more details on safety procedures, refer to the safety data sheet established in accordance with Regulation (EC) No. 1907/2006

- **Sodium hydroxide (NaOH) concentrated solution, 10 N (molar concentration)**

  Weight 40 g of sodium hydroxide anhydrous (*M = 40 g/mol*) into a 250 mL glass beaker (6.6) and add 50 mL of distilled water (5.6) and swirl to dissolve the sodium hydroxide powder. Dilute to 100 mL with distilled water (5.6) and mix.

- **Sodium hydroxide solution, 2N (molar concentration)**

Using a pipette, transfer 50 m**L** of concentrated sodium hydroxide solution (5.11) to a 250 m**L** one-mark volumetric flask (6.5). Dilute to the mark with distilled water (5.6) and mix.

6 APPARATUS

This SOP needs standard laboratory apparatus including:

- **Analytical balance**, capable of weighing to an accuracy of ± 0.01.
- **Water bath**, capable of being maintained at 95 °C ± 1 %.
- **Centrifuge**, capable of 5000 RCF (relative centrifugal force), approximately 6000 g.
- **Pipette**, automatic to cover the volume range (1, 5 and 10 mL) for extraction solution and sample dilutions.
- **One-mark volumetric flasks,** glass or plastic of capacities of 100 mL, 250 mL and 1 L.
- **One-mark volumetric beaker** glass or plastic of capacities of 250 mL.
- **Vortex mixer**, for efficient mixing during extraction.
- **Extraction tubes**, plastic, of 15 and 50 mL capacity with screw cap and able to withstand being centrifuged. Falcon® tubes are the most suitable.
- **Eppendorf® tube**, of 2 mL capacity to recover the final product of the extraction for colorimetric assay.
- **Vial**, glass of 2 mL capacity with screw cap to recover the final product of the extraction for HPLC-DAD analysis.
- **Syringe,** plastic of 2 mL capacity without needle to filtrate sample extract.
- **Filter,** a PTFE syringe type of 13 mm 0.45 µL to purify the sample during extraction.
- **Concentrator**, a centrifugal one capable to evaporate water and organic solvents including methanol and diethyl ether.
- **Spectrophotometer UV-Visible able to accommodate 100 path length cells**
- **Mechanical shaker**, Suitable for 50 mL centrifuge tubes for good sample homogenization and extraction efficiency.

7 PROCEDURE

7.1 Extraction of phenolic compounds

- Weigh approximately exactly 1 g of the freeze-dried powder sample into a 50 mL-extraction tube (6.8).

Extractable phenolic acid compounds

- Add to sample powder 5 mL of methanol/water extraction solvent (5.9). Mix using a vortex mixer (6.7) to extract the phenolic compounds.
- Keep the mixture (7.2) on a mechanical shaker (6.15) for 1 h at room temperature.

- Place the tube in the centrifuge (6.3) at 6000 g for 10 min at room temperature.
- Carefully decant the supernatant into a new 50 mL-graduated tube (6.8).
- Repeat extraction steps 7.1.2 to 7.1.5 two more times. Combine all extracts. Adjust the volume to 12 mL with methanol/water extraction solvent (5.9) and mix the contents. **This corresponds to the extractable *MeFPA* extract.**
- Partition equivalent weight of the pellet into two 50 mL centrifuge tubes, check the weight by weighing and keep the tubes at 4 °C until use for further extraction of bound phenolic acid components (7.1.26).
- Using filter (6.12) and syringe (6.11), filter two aliquots of 1 mL each of the MeFPA extract (7.1.6) into an Eppendorf tube (6.9) and a glass vial (6.10) for Folin Ciocalteu colorimetric assay and HPLC-DAD analysis, respectively.
- Keep both Eppendorf and vial tubes at -20 °C until FC colorimetric assay .
- Partition the 10 mL remaining MeFPA extract (7.1.6) in two aliquots (5 mL each) into two 50 mL centrifuge tubes (6.3).
- Evaporate completely the two aliquots (7.1.10) using a concentrator (6.13). Keep the tube at 4 °C until use for alkaline (7.1.12 - 7.1.19) and acid hydrolyses (7.1.20 - 7.1.25).

Extractable and alkaline-hydrolysable phenolic acid compounds

- Perform alkaline hydrolysis of extractable *MeFPA* (7.1.6) compounds. Re-dissolved the content of one tube (7.1.10) in 5 mL of 2N NaOH solution (5.12) and stirred for 4 h at room temperature.
- Acidify the alkaline hydrolyzed extract to pH 2 with concentrated HCl solution (5.2).
- Add 5 mL of Diethyl Ether (5.3). Mix using a vortex mixer (6.7).
- Place the tube in the centrifuge (6.3) at 6000 g for 10 min at room temperature.
- Carefully decant the supernatant into a new 50 mL-graduated tube (6.8).
- Repeat extraction steps 7.1.14 to 7.1.16 two more times. Combine all extracts and mix.
- Evaporate completely the Diethyl Ether (5.3) and re-dissolved the pellet in 2 mL of methanol/water extraction solvent (5.9). Mix using a vortex mixer (6.7) to dissolve the sample. **This corresponds to the *BHPA* (alkaline-hydrolysable phenolic acid) extract.**
- Repeat the step 7.1.8 to 7.1.9 to prepare *BHPA* extract for FC colorimetric assay.

Extractable and acid-hydrolysable phenolic acid compounds

- Perform acid hydrolysis of extractable *MeFPA* (7.6) compounds. Re-dissolved the content of the second tube (7.1.10) in 5 mL of 3M HCl (5.10).
- Place the extraction tube containing the sample (7.1.19) in the water bath set at 95 °C.
- Heat for 20 min the extraction tube in the water bath set at 95 °C, mixing on the vortex mixer every 5 minutes.

  Note: It is important to mix the samples thoroughly to ensure complete extraction.
- Remove the extraction tube from the water bath and allow it to cool down to room temperature.

- Repeat diethyl ether extraction steps 7.1.14 to 7.1.16 to obtain the ***AHPA* (bound acid-hydrolysable phenolic acid) extract.**
- Repeat steps 7.1.8 to 7.1.9 to prepare *AHPA* sample for FC colorimetric assay.

Bound and alkaline-hydrolysable phenolic acids

- Perform alkaline hydrolysis of bound phenolic acid. Re-dissolve the pellet contained in one tube (7.1.7) and store at 4 °C in 5 mL of 2N NaOH solution and stirred overnight at room temperature.
- Repeat extraction steps 7.1.14 to 7.1.16 to obtain the ***bBHPA* (bound alkaline-hydrolysable phenolic acid) extract.**
- Repeat extraction steps 7.1.8 to 7.1.9 to prepare sample for FC colorimetric assay.

Bound and acid-hydrolysable phenolic acids

- Re-dissolve the pellet contained in one tube (7.1.7) and store at 4 °C (7.1.7) in 5 mL of 3M HCl (5.10).
- Repeat the extraction steps 7.1.21 to 7.1.23 to perform acid hydrolysis of bound phenolic acids.
- Repeat extraction steps 7.1.14 to 7.1.16 to obtain the ***bAHPA* (bound acid-hydrolysable phenolic acid) extract.**
- Repeat steps 7.1.8 to 7.1.9 to prepare *bAHPA* sample for FC colorimetric assay.

7.2 Qualification of total phenolic acid compounds

Using 1 mL of *MeFPA* (7.1.9), *BHPA* (7.1.19), *AHPA* (7.1.25), *bBHPA* (7.1.28) or *bAHPA* (7.1.31) extract, determine the total PA compounds using a standard FC colorimetric method and Gallic acid calibration curve.

- PA quantification
  - Into a 10-ml screw tube, transfer 1 ml of PA extract (experimental tube) or methanol/water solvent (5.9; blanc)
    - Add 2.5 ml of FC
    - Mix and kept at room temperature for 3-5 min
    - Add 2ml of NaCO3 and mix
    - Incubated 30 min at room temperature
    - Measure the optical densities of experimental tube against blanc on the spectrophotometer (6.14).

      Note: Absorbance value should be included within the range of that of calibration curve. Otherwise, PA extract must be concentrated or dilute for lower and higher absorbance value, respectively.
- Standard calibration curve
  - Prepare a Gallic acid stock solution of 1mg/ml in water extract solvent (5.9),
  - Using the previous stock solution, transfer into 100ml mark tube 0, 1, 2, 3, 4, 5 ml of stock solution. Duplicate each tube.

- Dilute to the mark with water to obtained standard solutions with a final Gallic acid concentration of 10, 20, 30, 40, 50 µg/ml.

Note: Gallic acid standard solutions should be prepared daily

- Perform the FC colorimetric assay as previously described (7.2.1)

  Note: The reagent blank corresponding to Gallic acid concentration with and an optical density > 0.01 may indicated a contamination of FC stock solution, distillated water used to dilute the FC solution

- Based on the obtained absorbance data, construct the best-fit linear calibration graph from Gallic acid standards solution:

  $y = ax$

  Where

  y is the total phenolic acid content expressed as EGA(µg/ml)

  a is the slope value: EGA (µg/ml)/Absorbance

  x is the Absorbance

8 EXPRESSION OF RESULTS

8.1 Method of calculation and formulae

The total PA content (µg EGA/g DM) is calculated using the following formulae

$$PA = (Ae - Ab)\, x\, a \times D \times Ve$$

Where

Ae is absorbance of extract

Ab is absorbance of blanc

Ve is the initial volume of PA extract (12mL)

D is the dilution factor for a concentrated PA extract (7.2.1).

the suitable dilution factor (D) is the one that allows to have an absorbance included in the calibration range

8.2 Repeatability

The standard deviation between experimental repetitions carried out in a short time interval shall not exceed 10% of the average (coefficient of variation), data obtained under our experimental conditions on cassava (see appendix).

9 CRITICAL POINTS OR NOTE ON THE PROCEDURE

The quality of the sample to be analyzed (level of freeze-drying and grinding) can have a significant impact on the extraction efficiency. It must therefore be ensured that the sample is freeze-dried

properly and ground into a fine powder with a granulometry up to 250µm. The use of coffee grinder is sufficient to obtain a suitable powder of PA extraction.

During the alkaline hydrolysis step of the bound phenolic compounds, the suspension of the pellet in the alkaline solution is a delicate step that can impact the efficiency of the extraction. It would therefore be appropriate to dissolve progressively, with the pipette, the pellet in the NaOH solution. If necessary, the pellet can be dissolved in water and then diluted in a more concentrated alkaline solution so as to have a final NaOH concentration of 2N.

On should note that, the FC colorimetric essay is a global method that does not take into account possible interfering compounds. In order to avoid the potential bias, it is appropriate for each material either to evaluate the proportion of these interfering compounds in the total PA extract or separated and quantify specifically PS compound through HPLC analysis.

10 TEST REPORT

The test report must indicate the method used and the results obtained. In addition, any optional or experimental conditions not indicated in the SOP and any special circumstances that may have affected the results will be detailed.

The test report must include all details necessary for accurate sample identification;

11 APPENDIX

	Extractible total phenolic acid (µg EGA/g DM)					
	MeFPA		AHPA		BHPA	
Genotype	Locality 1	Locality 2	Locality 1	Locality 2	Locality 1	Locality 2
A	293.17 ± 17.7	165.8 ± 8.6	221.88 ± 9.1	154.77 ± 13.3	22.6 ± 0.3	13.8 ± 0.4
B	326.2 ± 4.2	331.2 ± 10.8	214.86 ± 14.3	155. 61 ± 3	16.6 ± 0.1	23.1 ± 2
C	297.65 ± 6.3	171.2 ± 11	195.75 ±19.3	207.13 ± 11.5	21 ± 2.2	11.2 ± 0.1
D	368.23 ± 11.1	372.1 ± 15.5	167.52 ± 15.8	210.76 ± 14.8	19.2 ± 2.2	22.1 ± 0.8
E	419.2 ± 16.5	486 ± 5.7	297 ± 5.5	365.5 ± 36.7	13.9 ± 0.7	12. 3 ± 0.9
F	658.37 ± 24	342.6 ± 17.3	191.53 ± 4.3	339.84 ± 4.6	12.8 ± 1.1	12.04 ± 0.6

	Bound total phenolic acid (µg EGA/g DM)			
	bAHPA		bBHPA	
Genotype	Locality 1	Locality 2	Locality 1	Locality 2
A	525.7 ± 35.3	673.5 ± 0.8	45.4 ± 3.1	38.6 ± 1.8
B	609.8 ± 3.6	642.5 ± 5.4	45.5 ± 0.2	87.5 ± 1.2
C	513.8 ± 13.5	597 ± 16.2	39 ± 1.7	50.82 ± 4.3
D	551.3 ± 1	595.2 ± 7	47 ± 0.0	50.7 ± 3.3
E	642.5 ± 19.5	695.2 ± 16	61.83 ± 2.0	103.7 ± 1.2
F	575 ± 23.4	430.6 ± 12.7	41 ± 2.4	99 ± 0.8

Table 1: Total phenolic content in different cassava fractions (µg EGA/g DM)

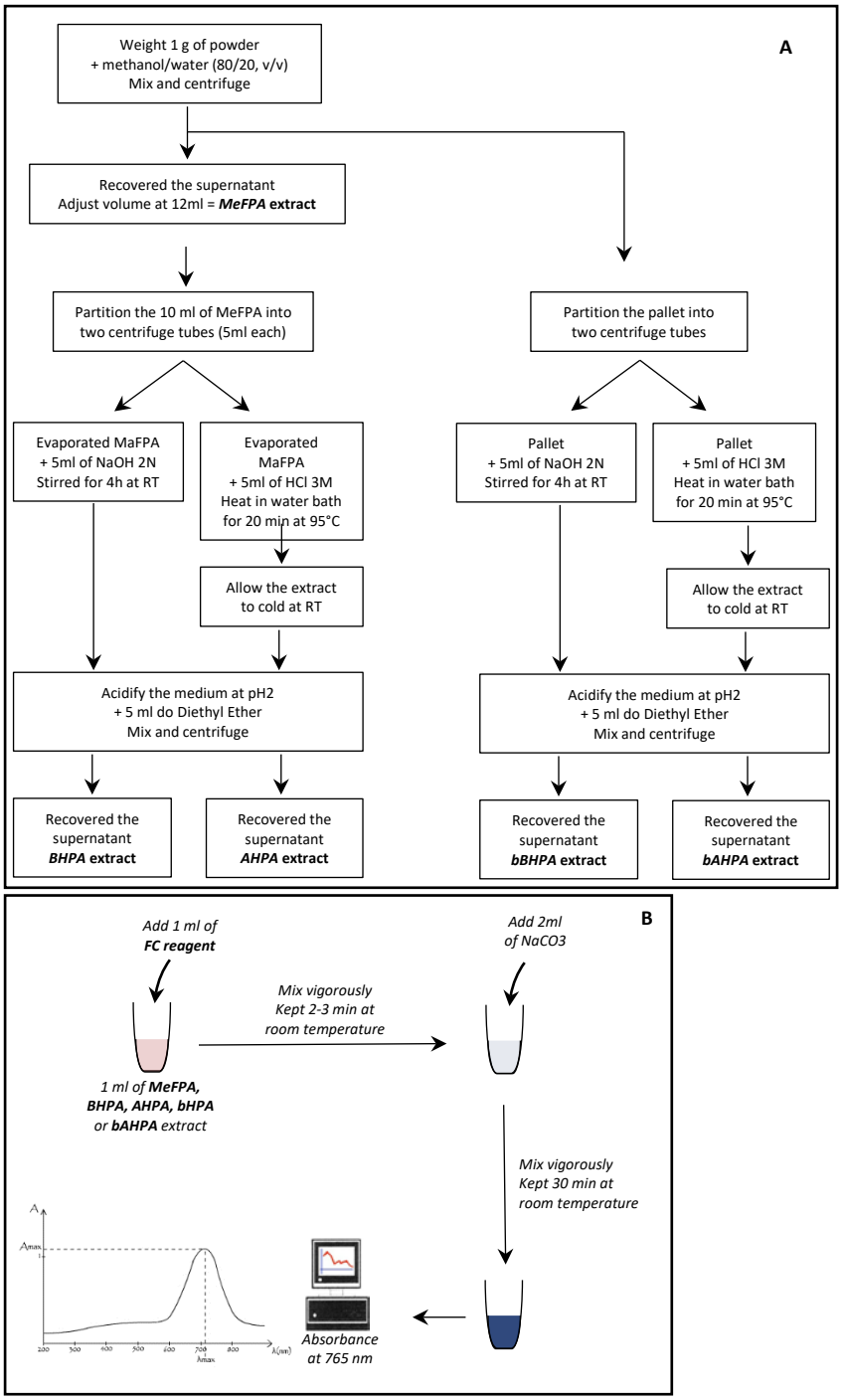

Figure 1: Flowchart of total phenolic acid extraction (**A**) determination procedure (**B**)

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/