

# Biochemical characterization of pulp of banana fruit: measurement of soluble sugars, organic acids, free ACC and *in vitro* ACC oxidase

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## Biochemical characterization of pulp of banana fruit: measurement of soluble sugars, organic acids, free ACC and *in vitro* ACC oxidase.

**Abstract — Introduction.** We present some protocols aiming at partially characterizing banana fruit quality through measurement of some key biochemical parameters. The principle, key advantages, starting plant material, time required and expected results are presented. **Materials and methods.** This part describes the required laboratory materials and the steps necessary for achieving four protocols making it possible to measure sugar, organic acids and free ACC contents, and *in vitro* ACC oxidase activity. **Results.** Standard results obtained by using the protocols described are presented in the figures.

France / *Musa sp.* / methods / fruits / measurement / quality

## Caractérisation biochimique de la pulpe de banane : mesure des sucres solubles, des acides organiques, de l'ACC libre et de l'ACC oxydase *in vitro*.

**Résumé — Introduction.** Nous présentons quelques protocoles visant à caractériser partiellement la qualité de la banane par la mesure de quelques paramètres biochimiques importants. Le principe, les principaux avantages, le matériel végétal requis, le temps nécessaire aux mesures et les résultats escomptés sont présentés. **Matériel et méthodes.** Cette partie décrit le matériel de laboratoire requis et les étapes nécessaires pour réaliser quatre protocoles permettant de mesurer les teneurs en sucre, acides organiques et ACC libre, ainsi que l'activité de l'ACC oxydase *in vitro*. **Résultats.** Les résultats standard obtenus en utilisant les protocoles décrits sont illustrés par des figures.

France / *Musa sp.* / méthode / fruits / mesure / qualité

## 1. Introduction

### Application

These protocols aim at partially characterizing banana fruit quality through measurement of some key biochemical parameters.

### Principle

Pulp of the fruit is cut into small pieces which are immediately frozen in liquid nitrogen. This fresh material is stored at  $-80\text{ }^{\circ}\text{C}$  until it is freeze-dried for sugar, organic acid and 1-amino-cyclopropane-1-carboxylic acid (ACC) measurements, or used for measurement of ACC oxidase activ-

ity. Sugars and organic acids are extracted with cold autoclaved distilled water and measured by HPLC. Free ACC is extracted with hot alcohol and its amount determined through its chemical transformation into gaseous ethylene. ACC oxidase enzyme is extracted in aqueous buffer with protection against enzyme degradation and its activity is determined by the production of gaseous ethylene after providing ACC as substrate.

### Key advantages

– These protocols are suitable for a wide range of dessert- and cooking-banana cultivars.

\* Correspondence and reprints

*Fruits*, 2008, vol. 63, p. 187–191  
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DOI: 10.1051/fruits:2008010  
www.fruits-journal.org

- Using fresh material allows an easier extraction, whatever the considered protocol, mainly at the step of homogenization of ground material with the extraction solution. However, it is more liquid nitrogen-consuming and more time-consuming in terms of blender cleaning.
- Using freeze-dried material leads to a less-risk storage of material, easier use of a blender and decreased consumption of liquid nitrogen; however, homogenization of ground material in aqueous solution is more difficult.

### Starting material

These protocols use freeze-dried pulp, except for measurement of ACC oxidase activity, which requires frozen material.

### Time required

Approximately 1 h is necessary for measurement of sugars from freeze-dried plant material; 1 h, for measurement of organic acids from freeze-dried plant material; 4 h, for measurement of free ACC from freeze-dried plant material; 3 h, for measurement of *in vitro* ACC oxidase activity from frozen plant material.

### Expected results

Yields are variable according to the cultivar and the developmental stage considered. Starting from 100 g of dry matter: 0–70 g of sugars, 2–6 g of organic acids and 0–3.5  $\mu\text{mol}$  of free ACC are expected; Starting from 100 g of fresh matter: 0–300  $\mu\text{L}$  ethylene- $\text{h}^{-1}$  for ACC oxidase activity can be obtained.

## 2. Materials and methods

### Laboratory materials

#### Materials for sugar measurements

For sugar measurements, the protocol requires:

- a coffee blender,
- distilled, autoclaved cold water,

- an Ultra-Turrax blender,
- distilled ultrapure water (Milli-Q Water system from Millipore),
- sterile 50-mL polypropylene centrifuge tubes,
- cloth filters to remove debris,
- duolite resin MB 6113 (Prolabo),
- C18 cartridge
- a glass pre-filter and 0.45- $\mu\text{m}$  nylon filter,
- methanol,
- HPLC fitted with RI detector (Waters 410), guard column sugar pak I (Waters), analytical column sugar pak I cation exchanger (Waters), ultrapure water as carrier liquid, Millennium 32 (Waters) as driving software.

#### Materials for measurement of organic acids

For measurement of organic acids, the same laboratory materials as for sugar measurements are required except that:

- duolite resin MB 6113 (Prolabo) is replaced with Dowex resin 50 W (Sigma),
- HPLC fitted with RI detector is replaced with a HPLC fitted with UV detector (Waters 486), analytical column C18 Spherisorb ODS2 (46  $\times$  250) (Waters), 2% ammonium dihydrogenophosphate pH 2.18 as carrier liquid, Millennium 32 (Waters) as driving software.

#### Materials for measurement of free ACC

For measurement of free ACC, the protocol requires: a coffee blender, a vortex, a sterile 50-mL Falcon tube, a microcentrifuge, cloth filters, ice, a heating water bath, 70% ethanol, distilled water, PEG 8000 (Poly Ethylene Glycol),  $\text{HgCl}_2$  (*Note*: a very toxic compound which must be handled according to specific safety rules), 2 N HCl, 2 N NaOH, NaOH in pellets, 15° NaOCl, 12-mL flask with a septum cap, CPG fitted with a flame ionization detector, 80–100 mesh activated alumina column, nitrogen as carrier gas.

#### Materials for measurement of ACC oxidase activity

For measurement of ACC oxidase activity, the protocol requires:

- liquid nitrogen, a coffee blender, PVP 10 (Poly Vinyl Pyrrolidone), a polystyrene

plate, a sterile 50-mL polypropylene centrifuge tube,

– a first solution (solution 1) for extraction and column equilibration: 100 mM tris HCl pH 7.5, 2 mM DTT, 30 mM sodium ascorbate, 10% glycerol (must be fresh and kept at 4 °C),

– ice, an agitating table, a refrigerated (4 °C) centrifuge that can accommodate 50-mL tubes, 0.45- $\mu$ m cellulose acetate filters, 12-mL sterile tubes (Falcon), a Sephadex G25 M column (PD-10, Pharmacia),

– a second solution (solution 2) for activity measurement: 100 mM tricine pH 7.5, 30 mM sodium ascorbate, 10% glycerol, 25 mM sodium bicarbonate, 125  $\mu$ M iron (II)-sulfate, 1.25 mM ACC (must be fresh),

– a 12-mL flask, a heating water bath, a vortex, CPG fitted with a flame ionization detector, 80–100 mesh activated alumina column and nitrogen as carrier gas.

## Protocols

### Protocol for measurement of sugars

#### • Step 1

To prepare fruit material, harvest fruit, remove peel, cut pulp into small pieces, freeze them in liquid nitrogen and store at – 80 °C until freeze-dried. When pieces are freeze-dried, store them at –20 °C.

#### • Step 2

To grind pulp material and extract sugars:

– weigh approximately 3 g of freeze-dried material,

– grind this material in the coffee blender,

– weigh 0.5 g in a sterile 50-mL polypropylene centrifuge tube,

– add 20 mL of distilled water and homogenize with the Ultra-Turrax blender for 1 min,

– centrifuge at 4 °C and 15 000 g for 10 min,

– recover the supernatant,

– this crude sample can be frozen for storage and further analysis.

#### • Step 3

To purify sample:

– filter the sample through a cloth filter to remove debris,

– activate the C18 cartridge phase with methanol and fill up with 2 g of Duolite resin, then filter the sample through it,

– filter through a glass pre-filter and 0.45- $\mu$ m nylon filter.

#### • Step 4

To measure the concentration of sucrose, glucose and fructose (modified from Célestine-Myrtil and Parfait [1]), sucrose, glucose and fructose of the purified sample are separated by HPLC; then, operating conditions are as follows: oven temperature 80 °C and carrier liquid 0.5 mL·min<sup>-1</sup>. Sugars are detected with the RI detector and quantified using the method of external standards.

### Protocol for measurement of organic acids

#### • Step 1

To prepare fruit material, harvest fruit, remove peel, cut pulp into small pieces, freeze them in liquid nitrogen and store at – 80 °C until freeze-dried. When pieces are freeze-dried, store them at – 20 °C.

#### • Step 2

To grind pulp material and extract organic acids:

– weigh approximately 3 g of freeze-dried material,

– grind this material in the coffee blender,

– weigh 0.5 g in a sterile 50-mL polypropylene centrifuge tube,

– add 20 mL of distilled water and homogenize with the Ultra-Turrax blender for 1 min,

– centrifuge at 4 °C and 15 000 g for 10 min,

– recover the supernatant,

– this crude sample can be frozen for storage and further analysis.

#### • Step 3

To purify sample:

– filter the sample through a cloth filter to remove debris,

– activate the C18 cartridge phase with methanol and fill up with 2 g of activated Dowex resin, then filter the sample through it,

– filter through a glass pre-filter and 0.45- $\mu$ m nylon filter.

Step 4. To measure the concentration of malic and citric acids (modified from Célestine-Myrtil and Parfait [2]), malic and citric acids of the purified sample are separated by HPLC. Operating conditions are as follows: oven temperature 37 °C and carrier liquid 0.7 mL·min<sup>-1</sup>. Acids are detected with the UV detector and quantified using the method of external standards.

#### Protocol for measurement of free ACC

##### • Step 1

To prepare fruit material, harvest fruit, remove peel, cut pulp into small pieces, freeze the pieces in liquid nitrogen and store at -80 °C until freeze-dried. When pieces are freeze-dried, store them at -20 °C.

##### • Step 2

To grind pulp material and extract ACC:

- weigh approximately 3 g of freeze-dried material,
- grind this material in the coffee blender,
- weigh 0.5 g of it in a 50-mL sterile Falcon tube,
- add 7 mL of 70% ethanol,
- warm the sample at 90 °C (closed tube) in a closed water bath for 45 min
- filter it through a cloth filter to remove debris,
- evaporate the sample at 100 °C (open tube) in an open water bath,
- to the dry residue, add 4 mL distilled water, 1 mL 10% PEG 8000 and 50 µL 2 N HCl,
- use the vortex for 1 min,
- centrifuge the solution at room temperature and 1 200 g for 10 min,
- neutralize it with 50 µL of 2 N NaOH,
- filter the sample through a cloth filter,
- the sample can be frozen for storage and further analysis.

##### • Step 3

To measure the amount of free ACC (modified from Lizada and Yang [3]):

- in a 12-mL vial, insert 300 µL distilled water, 100 µL 80 mM HgCl<sub>2</sub> and 500 µL sample,
- close the vial and keep it on ice,
- prepare a mixture of NaOH / NaOCl as follows: 3.3 g pellets of NaOH + 3 mL distilled water + 11 mL 15° NaOCl,

- start the reaction by adding 100 µL of the NaOH / NaOCl mixture; close the vial immediately and use the vortex for a few seconds,
- put the vial on ice for 2.5 min,
- use the vortex a second time,
- with a syringe, take 250 µL of gas out of the 12-mL vial through its septum and inject it into the GPC. Operating conditions are: oven temperature 110 °C, gas carrier 40 mL·min<sup>-1</sup>,
- ethylene is separated from other gases through the column described above. Operating conditions are as follows: oven temperature 110 °C and carrier gas 30 mL·min<sup>-1</sup>. Ethylene is detected with the flame ionization detector and quantified through a method of external standard.

#### Protocol for measurement of *in vitro* ACC oxidase activity

##### • Step 1

To prepare fruit material, harvest fruit, remove peel, cut pulp into small pieces, freeze them in liquid nitrogen and store them at -80 °C until use.

##### • Step 2

To grind pulp material:

- put a sterile 50-mL polypropylene centrifuge tube in a -80 °C freezer,
- put a spatula in liquid nitrogen to cool it,
- weigh 400 mg of PVP 10,
- cool a small polystyrene plate with liquid nitrogen,
- weigh 8 g of frozen pulp in this polystyrene plate,
- cool this material with liquid nitrogen,
- put this material (with no residual liquid nitrogen) in the prepared coffee blender,
- add the pre-weighed PVP 10,
- grind the mixture of fruit material and PVP 10 to obtain a fine powder.

##### • Step 3

To extract ACC oxidase enzyme (modified from Liu *et al.* [4]):

- weigh 5.25 g of the mixture of ground fruit material and PVP 10 in the 50-mL polypropylene tube,
- centrifuge the tube pre-cooled in a -80 °C freezer (use the cooled spatula),
- add 20 mL of the 'solution 1' and carry on all the further steps at 4 °C,

- homogenize the mixture by gentle shaking until the slurry has completely thawed,
- put the tube on ice and shake gently on an agitating table for 15 min,
- centrifuge it at 30 000 *g* and 4 °C for 20 min,
- equilibrate a Sephadex G25 M column (PD-10, Pharmacia) with 25 mL of ‘solution 1’,
- recover the supernatant and put it on ice,
- pass 3 mL of supernatant through a 0.45- $\mu$ m cellulose acetate filter (you may need two filters according to the stage of development of the fruit, because of viscosity),
- de-salt by passage through the equilibrated Sephadex G25 M column.

• Step 4

To measure ACC oxidase activity (modified from Liu *et al.* [4]):

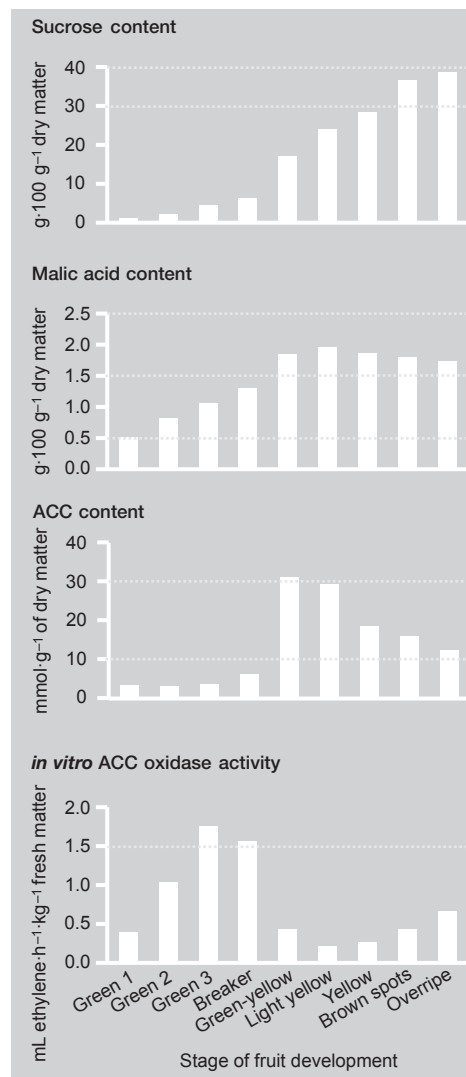
- in a 12-mL flask, put 800  $\mu$ L of ‘solution 2’,
- add 200  $\mu$ L of enzymatic sample and immediately close the flask,
- use the vortex for a few seconds and incubate the flask at 30 °C for 30 min,
- use the vortex again for a few seconds,
- with a syringe, take 250  $\mu$ L of gas out of the 12-mL flask through its septum and inject into the GPC,
- ethylene is separated from other gases through the column described above; operating conditions are as follows: oven temperature 50 °C and carrier gas 30 mL·min<sup>-1</sup>. Ethylene is detected with the flame ionization detector and quantified through a method of external standard.

**Troubleshooting**

One problem can occur: it is impossible to filtrate the crude sample after extraction whatever the protocol used. This can be due to a too large viscosity of the sample, which depends on the stage of fruit development. *Solution:* increase the volume of extraction solution versus the weight of plant material.

**3. Typical results obtained**

The different contents obtained with the measurement of biochemical parameters according to the stage of fruit development look like those presented in *figure 1*.



**Figure 1.** Changes in malic acid, sucrose, ACC (acid 1-aminocyclopropane-1-carboxylic) and *in vitro* activity of ACC (acid 1-aminocyclopropane-1-carboxylic) oxidase during development of banana fruit.

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