Laboratory Standard Operating Procedure

SOP for Sugars by HPLC

Montpellier, France, November 2019

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

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

To be cited as:

Christian MESTRES, (2019). SOP for *Sugars by HPLC.* Montpellier, France: RTBfoods Project Report, 10 p.

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: Sugars by HPLC

Date: 29/11/2019	**Release: 1**

Written by : Christian MESTRES

For information on this SOP please contact :

- Christian MESTRES (chirstian.mestres@cirad.fr)

This document has been approved by:

Partner	Name of the person who approved	Date
FSA/UAC	Noël AKISSOE	30/11/2019
CIAT	Thierry TRAN	01/12/2019

CONTENTS

Table of contents

1 SCOPE AND APPLICATION

The procedure describes the extraction (at ambient temperature) and assessment of sugars by HPLC in RTB crops (Roots, tubers and bananas) after extraction with acidified water.

Alcohols (ethanol and methanol) and organic acids contents can also be assessed with this procedure.

2 REFERENCES

MESTRES, C., ROUAU, X. **1997.** Influence of natural fermentation and drying conditions on the physicochemical characteristics of cassava starch. *Journal of the science of food and agriculture* **74**, pp. 147-155.

3 DEFINITIONS

Sugar is a term for a class of edible crystalline carbohydrates (with the empirical formula $C_m(H_2O)_n$) characterized by a sweet taste. The procedure can be used to assess simple sugars (glucose, fructose for example), and several di-holosides (sucrose and maltose).

An organic acid is an organic compound with acidic properties. The most common organic acids are the carboxylic acids, whose acidity is associated with their carboxyl group –COOH. Organic acids generally encountered in RTBs are first those originating from physiology (oxalic, citric etc) and then the ones generated during processing (acetic, lactic)

4 PRINCIPLE

Soluble sugars and organic acids are extracted at ambient temperature in aqueous medium in conditions inhibiting enzymatic activities and limiting chemical degradation (pH < 3). Extracted metabolites are then separated by HPLC on ionic and size exclusion column (for example: Aminex HPX87H-Biorad, Hercules, USA) **thermostated** at **35°C**. They are eluted with 5 mM sulfuric acid at 0.6 ml/min. Sugars are assessed by refractomery and organic acids by spectrophotometry at 210 nm. Compounds are identified by their retention time and quantified by their peak area (by comparison with standards). The double detection allows some validation of the identification: a sugar will only be detected by refractometry and the ratio between refractometric and spectrophotometric responses is known for each organic acid. Results are calculated in mg/g, dry basis.

5 REAGENTS

- 0.5 mM sulfuric acid
- Ultrapure or bi-distilled water, filtrated through 0.45 µm (**freshly prepared**)
- Eluent: **5 mM sulfuric acid**. Dilute 100 time 0.5 M sulfuric acid with ultrapure water. To be prepared just before use: **keep for a maximum of 48 H**.

- Standards of the sugars, organic acids and alcohol most commonly encountered are presented in table 1. This list should be adapted depending on the nature of the product to be analysed.

Table 1: Standards of sugars, organic acids and alcohol

Sugars	maltose sucrose glucose fructose
organic acids	oxalic citric lactic acetic
Alcohol	ethanol methanol

6 APPARATUS

- HPLC system consisting of a degasser, a pump, injector, a column and two detectors: ultraviolet spectrophotometer, and refractometer.
- Column exclusion of ions for analysis of sugars and organic acids: for example, Aminex HPX - 87 H column, 300 mm x 7.8 mm (Biorad, Hercules, USA).
- Guard-Column of the same nature.
- 20 µL injection loop
- 2 mL extraction/centrigugation tubes with « caps lock » caps (Ependorf for example)
- Rotary shaker (type agitest 34050, Bioblock)
- Centrifuge (type Genofuge, 24 D Legallais)
- filter syringes (pore size 0,45 µm)
- 1 mL Syringes and vials
- Balance (sensitivity : 0.1 mg)
- Vortex agitator

7 PROCEDURE

7.1 Extraction

- Prepare extraction and eluting solution (5 mM H_2SO_4,)
- Weigh 25 mg of powdery sample directly into a 2 mL tube (depending on the levels of organic acids and sugars in the product, this amount may be modified)
- Add 1 mL of extraction solution and agitate vigorously by placing on Vortex agitator
- Put extract on the Rotary Shaker for **1 h at room temperature**
- Centrifuge at 10 000 g for 5 min
- Filter 0.5 ml of supernatant with filter syringe (0. 45µm); put the filtrate (extract) directly into a 1 ml vial.
- Depending on the concentration of sugars and organic acids of the analyzed material, the filtrate can be diluted (with the extraction solvent) prior to analysis.

7.2 Solution of the standards of sugars and organic acids

Depending on the expected sugars and organic acids, injections of standard solutions (generally between 1 and 10 mg/ml) should be performed. An example of sugars and organic acids with the elution order and coefficients of response for both types of detectors is given in Table 2. Several standards can be injected together providing their retention times differ by at least 2 minutes, approximately. Note that methanol and ethanol can be determined by this procedure.

Table 2: Order of Elution of anions and sugars, with order of magnitude of response factors.

Compound		RT (min)	Response coefficient [surface /concentration mg mL^{-1}]	
			Refractometry	UV 210 nm
sugars	maltose	8	23.2	0
	saccharose	8.2	24.4	0
	glucose	9.6	24.7	0
	fructose	10.7	24.8	
organic acids	oxalic acid	6.6		414
	citric acid	8.4	21.6	35.3
	lactic acid	13.5	16.8	24.6
	acetic acid	15.4	11.3	17.1
alcools	methanol	19.4	~ 10	0
	éthanol	22.2	9.9	0

7.3 Chromatographic conditions

Mobile phase : 5mM Sulfuric acid
Flow rate : 0.6 mL/min
Injection volume : 20 µL
Column oven : 35°C
UV Detector wavelength : 210 nm

8 EXPRESSION OF RESULTS

8.1 Method of calculation and formulae

Identify each compound on the basis of its retention time and compare it to that obtained for the standard; retention times must not differ by more than 10 percent for reliable identification. On the other hand, this identification can be confirmed by observing the profiles on the two detectors (UV210 nm and refractometry); sugar or alcohol detected by refractometry should not attain a peak with similar retention time at UV210 nm.

Compound content (mg/g dry matter) = (A * f * 100000) / (RC *W * DM)

Where

- A is sample peak area
- f is the dilution factor
- RC is the response coefficient of the standard (mg/ml)
- W is sample weight in mg (wet basis)
- DM is the dry matter content (% wet basis) of the sample

In the case of organic acids, the average value of the levels assessed by the two detectors (UV210 nm and refractometry) is calculated. If the value calculated by absorbance at 210 nm is much higher (**more than double**) than that calculated by refractometry, it means that another compound highly absorbing in the UV domain is masking the organic acid. In this case, the organic acid must thus be considered to be **absent.**

8.2 Repeatability

The standard deviation between repetitions within a short time interval shall not exceed 5% of the average (coefficient of variation).

9 Critical Points or Note on the Procedure

- ✓ When the HPLC system is switched on, it takes several hours for the refractometer to stabilize; therefore, switch on the elution system several hours before starting a sequence of analysis, and rinse the cell reference of the refractometer each day with the eluent (5 mM sulphuric acid). When doing so, wait approximately ¼ hour to stabilize the baseline before auto-zero the refractometer.
- ✓ Check carefully the identification of compounds by joint observation of the two detectors (see 8.1)

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 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 for the sample.

11 REVISION RECORD

Date (DD/MM/YYYY) (arial 11 bold)	Responsible person	Description of change

Institution: Cirad – UMR QualiSud

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

Contact Tel: +33 4 67 61 44 31

Contact Email: rtbfoodspmu@cirad.fr