Laboratory Standard Operating Procedure

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SOP for the determination and quantification of phenolic compounds in bean flour by HPLC

Nutrition Quality Laboratory (NQL)

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Laboratory Standard Operating Procedure

SOP for the determination and quantification of phenolic compounds in bean flour by HPLC

Nutrition Quality Laboratory (NQL)

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Contents

1 Scope and application

This SOP describes the stages of the extraction process, drying, identification, and quantification of 9 of the main phenolic compounds in common beans (*Phaseolus vulgaris L.*) using high-performance liquid chromatography (HPLC):

- 3,4-dihydroxibenzoic acid
- (+)-Catechin
- Myricetin 3-glucoside
- Quercertin 3-glucoside
- Rutine
- Kaempferol 3-glucoside
- Myricetin
- Quercetin
- Kaempferol

The protocol for the determination and quantification of phenolic compounds in bean flour is also described in Lin et al. (2008): The polyphenolic profiles of common bean (*Phaseolus vulgaris* L.). *Food Chemistry*, 107(1), 399–410. https://doi.org/10.1016/j.foodchem.2007.08.038

2 Principles and definitions

Phenolic compounds comprise different families of secondary metabolites with different chemical structures, found in a huge diversity of food matrices, such as pulses, fruits, vegetables, and cereals (Albuquerque et al., 2021). The biological activity of these compounds plays an important role in human health. Several studies report that the consumption of pulses can improve metabolic markers normally associated with a variety of non-communicable diseases, such as diabetes mellitus, hypertension, obesity, among others. These potential health benefits have been attributed to the presence of phenolic compounds that possess antioxidant properties (Alfaro-Diaz et al., 2023).

Common beans (*Phaseolus vulgaris L.*) contain a wide range of phenolic compounds, present in cotyledons and seed coats, with different phenolic composition between varieties that could be related to seed color (Rodriguez Madrera et al., 2021). Bean cotyledons have abundant phenolic acids (ferulic, protocatechuic, aldaric, and *p*-coumaric), while in the coats, there are different flavonoids, including flavonols (kaempferol, quercetin, and myricetin), flavanols (catechin and epicatechin), glycosylated flavonoids (rutin and 3-*O*-glycosides of kaempferol, quercetin, and myricetin), and anthocyanins (delphinidin, petunidin, or malvidin, 3-*O*-glucosides) (Lin et al., 2008; Alfaro-Díaz et al., 2023).

The most widely used method to determine individual phenolic compounds in beans and correlate the concentration of these molecules with seed color and antioxidant activity is high-performance liquid chromatography (HPLC) coupled with diode array detection (DAD) or mass spectrometry (MS) (Giusti et al., 2019; Lin et al., 2008; Luthria & Pastor-Corrales, 2006). This method is based on the extraction of the compounds of interest with an 80% methanolic solution. Subsequently, the extract is concentrated to dryness and finally reconstituted with 80% methanolic solution to be analyzed by HPLC-DAD technique.

3 Apparatus

Pipettes: 1 mL and 5 mL

Centrifuge tubes: 2 mL

Flasks: 250 mL, 1000 mL, and 2000 mL

Beakers: 100 mL

Graduated cylinder: 250 mL

Vortex: Mixer/homogenizer, multivortex

Balance: Analytical, readability 0.1 mg

Centrifuge: >6000 RCF (relative centrifugal force)

Ultrasonic bath: >60 Hz frequency, >60 Watts power

Rack: For coupling centrifuge tubes and vials

Vacuum concentrator system: With temperature control (25–80°C)

Fume hood or extraction cabin: At least 100 fpm (feet per minute)

Chromatography column: Phenomenex Synergy 4µ Hydro-RP 80Å, LC column 4.6 x 150 mm

Guard column: SecurityGuard Phenomenex KJ0-4282

Quaternary pump: Agilent 1200 HPLC quaternary pump

Autosampler: Agilent 1200 HPLC autosampler

Detector: Agilent 1200 HPLC diode array detector 200–900 nm

4 Reagents and standards

Methanol: HPLC grade (CAS number 67-56-1)

Formic acid: Standard grade (CAS number 64-18-6)

Acetonitrile: HPLC grade (CAS number 75-05-8)

(+)-Catechin: Analytical or standard grade (CAS number 154-23-4)

3,4-dihydroxibenzoic Acid: Analytical or standard grade (CAS number 99-50-3)

Kaempferol: Analytical or standard grade (CAS number 520-18-3)

Kaempferol 3-glucoside: Analytical or standard grade (CAS number 480-10-4)

Quercetin: Analytical or standard grade (CAS number 117-39-5)

Quercetin 3-glucoside: Analytical or standard grade (CAS number 482-35-9)

Myricetin: Analytical or standard grade (CAS number 529-44-2)

Myricetin 3-glucoside: Analytical or standard grade (CAS number 19833-12-6)

Rutin: Analytical or standard grade (CAS number 207671-50-9)

Water: Type I (0.056 µS/cm to 25°C max conductibility)

5 High-Performance Liquid Chromatography (HPLC) conditions

Flow: 1.0 mL/min

Column temperature: 30 ± 1 °C

Run-time: 60 min

Post-run time: 5 min

Injection volume: 20 µL

Mobil phase A: Formic acid in water 0.1%

Mobil phase B: Formic acid in acetonitrile 0.1%

Wavelength detection: DAD read at 260 nm, 280 nm, 370 nm, and 520 nm

Gradient variation phase:

1st ramp. Linear variation from 10–26% B (v/v) in 40 min

2nd ramp. Linear variation to 65% B (v/v) at 55 min

3rd ramp. Linear variation to 90% B (v/v) at 60 min

4th ramp. Held at 90% B (v/v) to 60 min

5th ramp. Post-run 10% B (v/v) at 61 min and held to 65 min

6 Movil phase solutions and extraction solution preparation

Mobil phase A

To prepare 1000 mL of 0.1% formic acid, take an aliquot of 1.14 mL of formic acid (98% v/v) and bring to volume in a 1000 mL volumetric flask with water type 1.

Mobil phase B

To prepare 1000 mL of 0.1% formic acid in acetonitrile, take an aliquot of 1.14 mL of formic acid (98% v/v) and bring to volume in a 1000 mL volumetric flask with acetonitrile.

Methanol extracting solution (80% v/v)

To prepare 250 mL of 80% methanol extracting solution, measure 200 mL of methanol (99%, analytical grade) in a graduated cylinder and transfer it to a 250 mL volumetric flask. Bring the volume to 250 mL with water type 1.

7 Preparation and calculations of calibration curves

7.1. Preparation of standards and calibration curves

The steps in this section apply to the calibration of all phenolic compounds of interest (3,4-dihydroxibenzoic acid, (+)-catechin, myricetin 3-glucoside, quercertin 3-glucoside, rutine, kaempferol 3-glucoside, myricetin, quercetin and kaempferol) to cover the range between 2–120 µg/mL (view Example 1).

Example 1

Prepare 10 mL of a concentrated stock solution standard of approximately 400 µg/mL of standard (3,4-dihydroxibenzoic acid).

Note: The final weight of the solution standard will change based on the purity of the standard (3,4-dihydroxibenzoic acid).

The calculation procedure to determine the amount of standard to use for S1 solution, assuming a purity of 99.6%, is shown in Equation 1.

Equation 1:

$$10\ mL\ stock\ sol. \times \frac{400\ \mu g\ 3{,}4-dihydroxibenzoic\ a.}{1\ mL\ stock\ sol.} \times \frac{100\ \mu g}{99.6\ \mu g\ dihydroxibenzoic\ a.}$$

$$= 4020\ \mu g\ (or\ 4.020\ mg)$$

Based on the example above, weigh 4.02 mg of 3,4-dihydroxibenzoic acid (assuming 99.6 % purity) and transfer it to be diluted in a 10 mL volumetric flask with methanol (80% v/v). Label this solution as STOCK (S1).

Note: When using a balance with resolution/precision of 0.1 mg, typically found in laboratories, the target to weight for this specific example will be 4.0 mg.

Prepare the calibration curve standards as described in Table 1.

Table 1. Preparation of 3,4-dihydroxibenzoic acid calibration curve from SA and SB solution.

Solution ID	Target 3,4-dihydroxibenzoic acid concentration (µg/mL)*	Volume of solution ID needed (mL)	Take from solution ID (mL)	Volume of methanol 80% needed (mL)	Final volume (mL)
SA	120.1	3.0	S1	7.0	10.0
SA1	90.1	1.5	SA	0.5	2.0
SA2	60.1	1.0	SA	1.0	2.0
SB	40.0	1.0	S1	9.0	10.0
SB1	30.0	1.5	SB	0.5	2.0
SB2	20.0	1.0	SB	1.0	2.0
SB3	10.0	0.5	SB	1.5	2.0
SB4	4.0	0.5	SB	4.5	5.0
SB5	2.0	0.25	SB	4.75	5.0

* Actual concentration of solutions to be injected into HPLC will vary based on a standard purity of 99.6% and actual weight of standard used.

Prepare the calibration curves of the other phenolic compounds in the same way, considering the purity of the compound of interest.

7.2. Obtaining the calibration curve equation

Create a plot of instrument response signal (peak area at target wavelength) versus known concentrations of the standards of interest as shown in Example 2 for 3,4-dihydroxibenzoic acid (Table 2). Determine the linear regression equation for the calibration curve of the compound of interest, for the target concentration segment (Fig. 1).

Example 2

Table 2. Response signal (Area) data at different known concentrations of 3,4-dihydroxibenzoic acid.

Solution ID	3,4-dihydroxibenzoic acid concentration (µg/mL)*	Area absorbance max (260 nm)
SA	119.52	7591.279
SA1	89.64	5667.337
SA2	59.76	3797.642
SB	39.84	2580.374
SB1	29.88	1937.411
SB2	19.92	1301.328
SB3	9.96	663.301
SB4	3.98	262.552
SB5	1.99	133.008

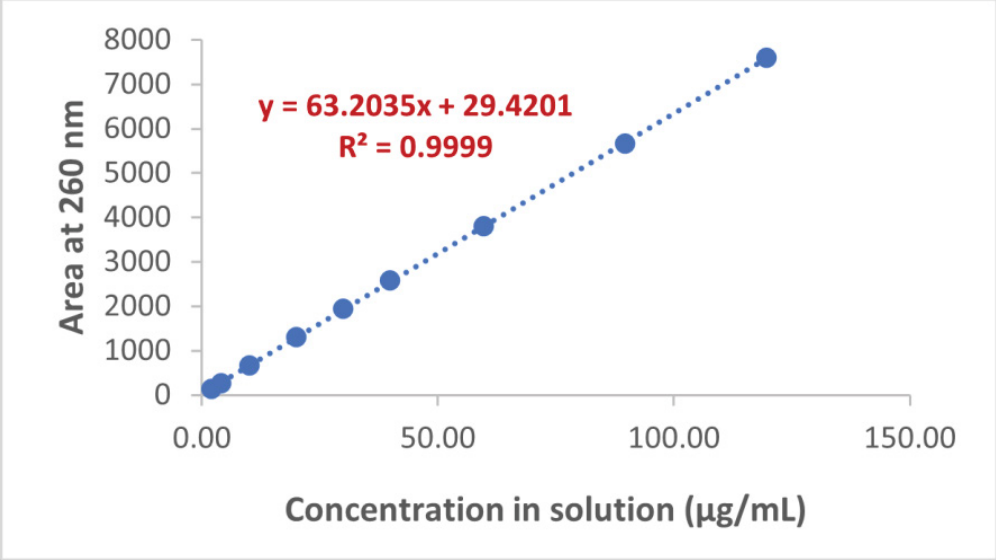

Figure 1. Calibration curve of 3,4-dihydroxibenzoic acid from data Table 2.

Ensure that the limit of detection for each calibration curve is low enough to cover the concentration expected in the routine samples.

8 Procedure

8.1. Sample preparation

The step of selection, washing, and milling of bean grains is described in SOP ID: NQL-2023-001 (sub-section 7.1) (Orozco et al., 2024).

8.2. Extraction

Add 0.2 g of the ground homogenized sample, to a 2 mL tube (Fig. 2).

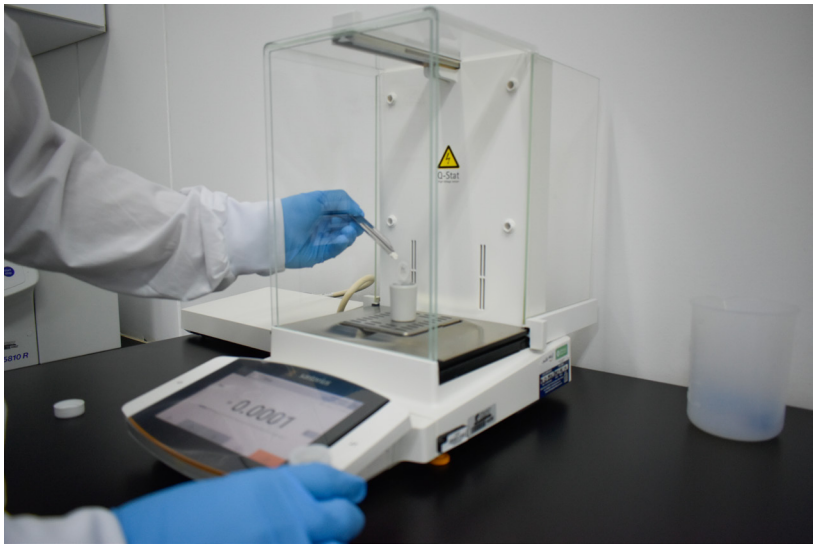

Figure 2. Sample weighing.

Add 1 mL of 80% (v/v) methanol solution to the tube containing the sample and vortex for 20 seconds, ensuring that the entire sample is exposed to the extracting solution (Fig. 3a). Then put the tube in the multivortex for 1 hour (Fig. 3b).

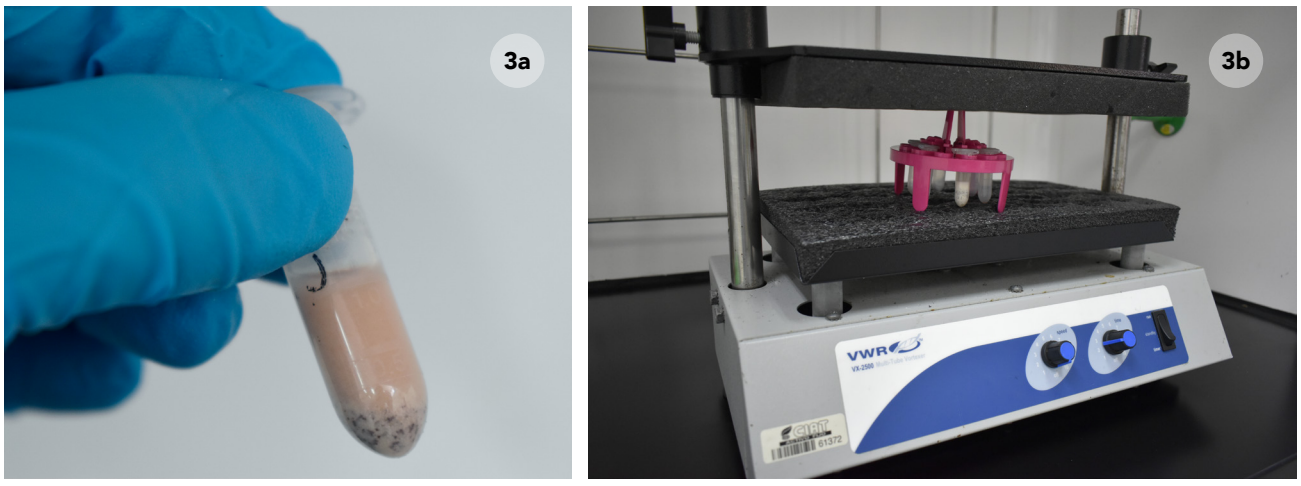

Figure 3. Adding 1 ml of extracting solution (3a). Vortex mixer with samples (3b).

Centrifuge at 6000 RCF for 15 minutes at room temperature (Fig. 4a). Transfer manually the supernatant to a clean, dry 2 mL tube (Fig. 4b).

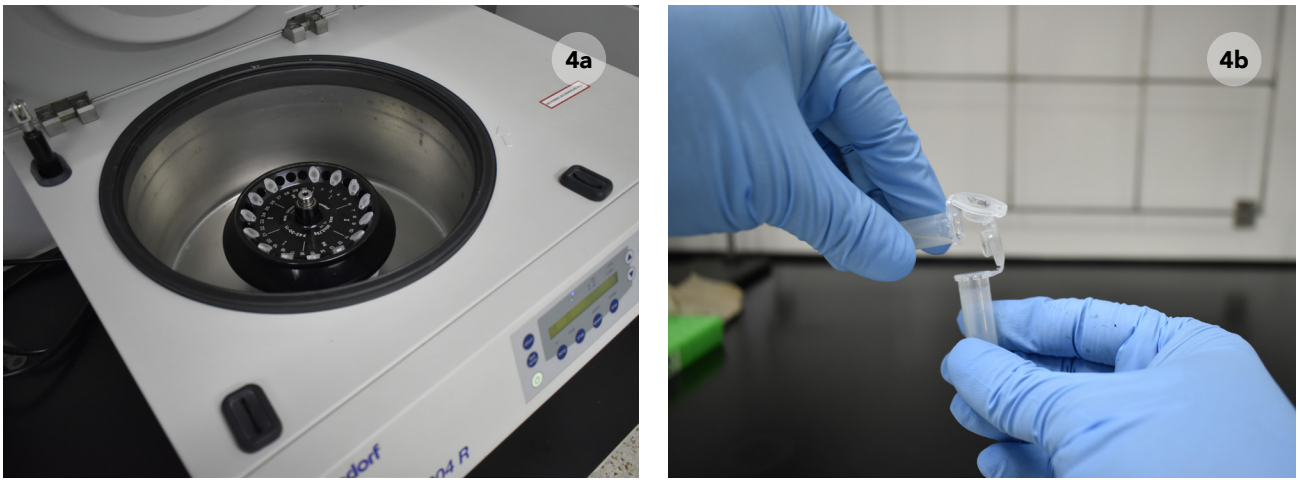

Figure 4. Centrifuging samples (4a). Transferring the supernatant manually (4b).

Repeat the extraction process two more times, always transferring the supernatant to the same collection tube.

8.3. Concentration and reconstitution of the extract

For the concentration process, place the tube (with lid open) with the supernatant obtained from the first extraction on the rotating rack of the vacuum concentrator (Fig. 5a). Start the vacuum concentration process by conditioning the equipment at 50°C / 0.005 mbar, and concentrate until dry (Fig. 5b).

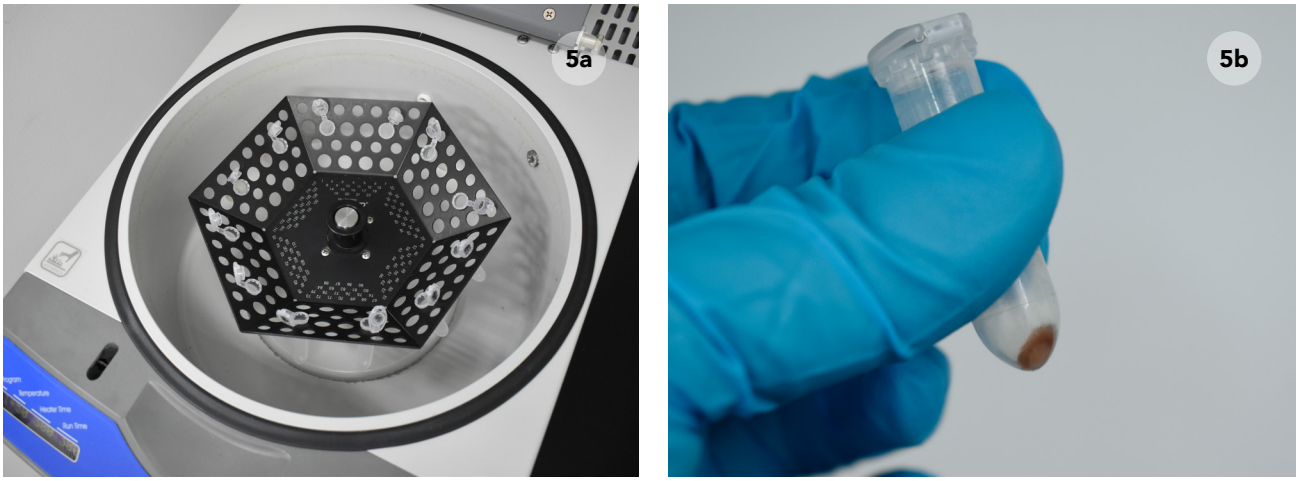

Figure 5. Sample placed in vacuum concentrator (5a). Completely dry extract (5b).

Note: While the supernatant from the first extraction is being concentrated, the laboratory technician can begin the process of the second/third extraction (last stage of step 8.2).

For the reconstitution of the extract, add 0.3 mL of methanol (80% v/v) to the tube with the extract and sonicate for 1 min (Fig. 6).

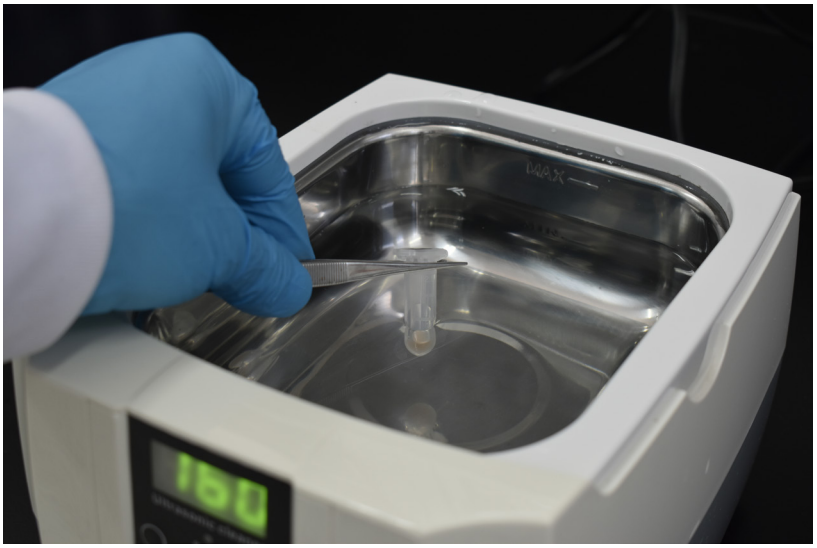

Figure 6. Position of the tube with the extract to be sonicated.

ALERT! An operating ultrasound bath should not be immersed in or touched by hand, as exposure to ultrasound can cause damage to the body, including burns, alterations in the nervous system, and cellular damage. It is important to wear protective gloves and glasses when working with an ultrasound bath, even if it is turned off.

Then vortex for 1 min until the dry extract is dissolved and a homogeneous mixture is obtained (Fig. 7a and Fig. 7b).

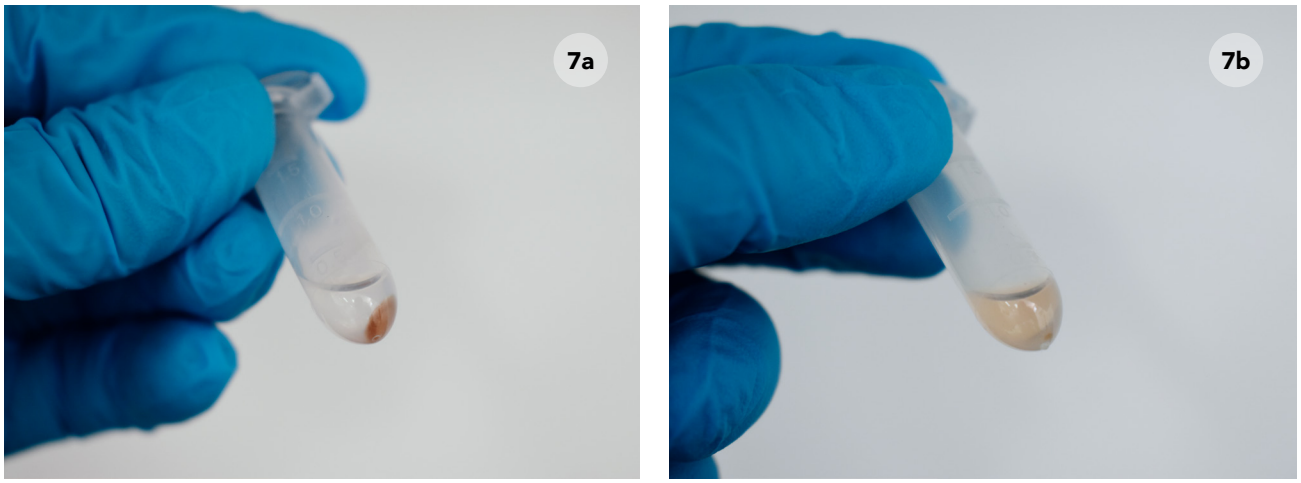

Figure 7. Extract with 0.3 mL of methanol before sonication and vortexing (7a). Extract reconstituted correctly after sonication and vortexing (7b).

8.4. Vial preparation

Once the extract is reconstituted, filter the solution into an insert vial for HPLC by using 0.22µm PTFE syringe filter (8a). Then, put the insert vial in a vial for HPLC and place it on the HPLC sample tray (8b).

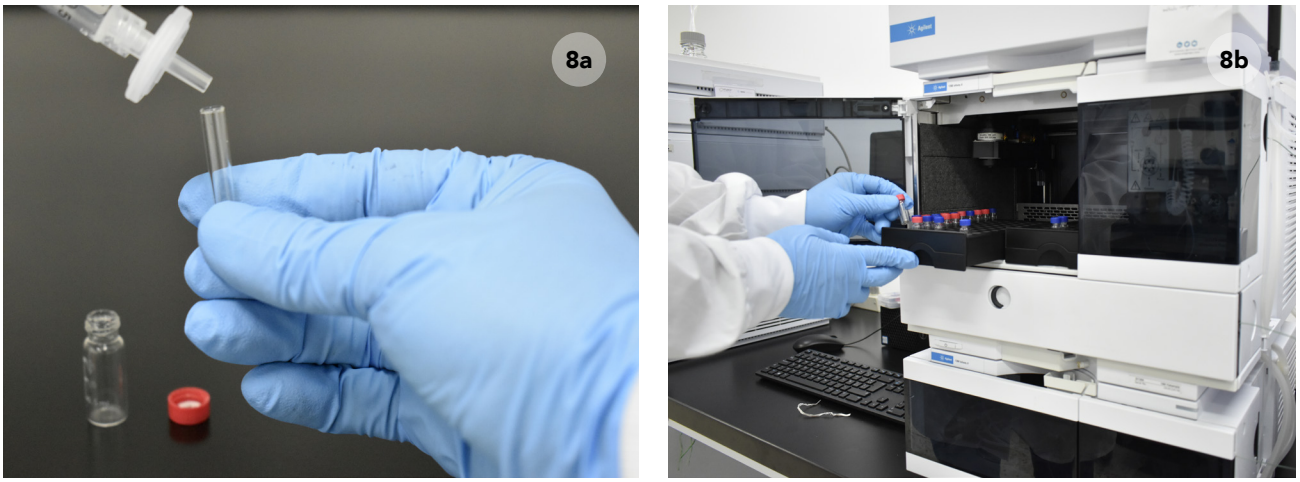

Figure 8. Filtration of the reconstituted extract into the insert vial (8a). Loading the vial into the HPLC system (8b).

8.5. HPLC chromatogram

Once the sample extract has been analyzed by the HPLC, a chromatogram (or graph) is generated containing the data collected by the HPLC detector (DAD UV-Vis) through the data acquisition software. This graph displays several peaks, where the area under each peak is proportional to the amount of the corresponding compound in the sample. A larger peak area indicates a higher concentration of the analyte. The peak area value is essential for calculating the analyte's concentration in the sample (Fig. 9).

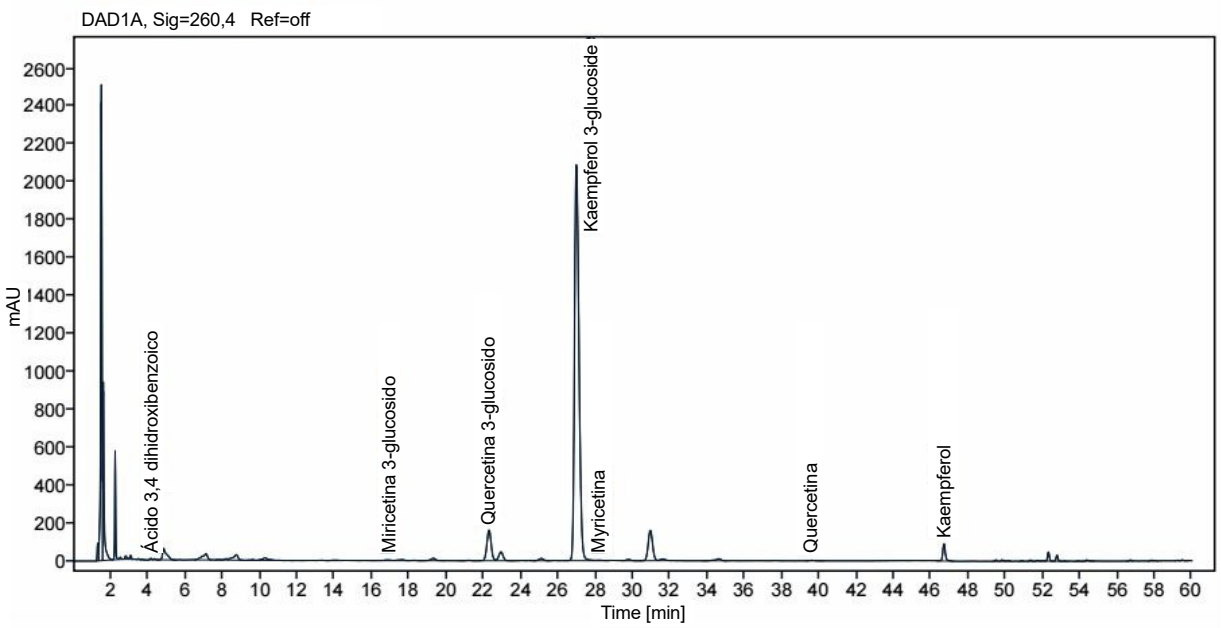

Figure 9. Typical chromatogram of a bean flour extract at 260 nm.

9 Calculation

Determine the concentration of phenolic compounds in the sample using a calibration curve and chromatogram, according to Equation 2.

Equation 2:

$$Compound\ concentration\ (\frac{\mu g}{mg}) = \left(\frac{Area\ (AUC) - intercept}{Slope\ (AUC/(\frac{ug}{ml})}\right) \times \frac{vol.reconstitution\ (mL)}{weigth_{sample}(mg)}$$

Where:

Compound concentration is the quantity of the compound being measured (µg) in each unit (mg) of sample tested, equivalent to mg of compound per g of sample.

Area is the value of the area under the peak of the compound in the chromatogram. Its units are expressed as "area under the peak" or "area under the curve" (AUC).

Intercept is the value of the intercept obtained in the calibration curve of the compound.

Slope is the value of the slope obtained in the calibration curve of the compound.

Vol. reconstitution is the volume (in mL) used to reconstitute the dry extract.

Weight sample is the weight (in mg) taken from the sample for analysis.

10 Quality control

To carry out the analysis on the samples, they must be taken in duplicate each 10 samples if the precision/ uncertainty level is < 5.0%, each 5 sample if the precision is between 5.0–10.0%, each 3 samples if precision is between > 10.0 ≤ 15.0% and a duplicate for each sample if precision is > 15%. If the coefficient of variation of the duplicate of the total content of the quantified phenolic compounds is less than 10%, it is considered a good value.

Phenolic compounds can degrade through direct photodegradation, which can be caused by exposure to UV, visible, and solar light. Verify that during the extraction, drying and reconstitution process there is NO direct light incidence on the extracts. If so, protect them from direct light with aluminum foil or use 2 mL centrifuge tubes of amber material.

Internal control sample: Analyze an internal control sample (in duplicate) every day of analysis. To prevent contamination (and moisture migration) of internal control samples, take several aliquots of the grain and put each in high barrier bags or amber glass bottles; finally place all aliquots in the same primary bag for storage (-20°C).

Discard samples if visible color particles are seen in the syringe filter disk, because this would highly likely indicate that the extract was not properly dissolved.

11 Disposal of waste and cleaning of material

The waste obtained after the extraction, separation, and elution process of the total phytates can be discharged through the pipeline normally. The solid part is collected on a paper napkin and deposited in the garbage can.

All waste obtained from the phenolic compound by HPLC procedure (samples and standards) must be collected in glass containers, labeled with the format of the department of health and safety at work and list the substances that are part of the mixture. Once the bottle is full, it must be taken to the hazardous waste warehouse at the times established by the department of health and safety at work, who manage hazardous waste. Syringes and filters contaminated with methanol should be placed in a container properly marked as waste hazardous.

Finally, the material used in the analysis must be placed in the ultrasonic bath for 15 minutes and then washed with citranox or alconox liquid soap and rinsed with distilled water from the network.

12 Annexes

Table 3. Reference wavelength (nm) and retention time (min) used in chromatography to identify and quantify phenolic compounds.

Phenolic compound	Λ(nm)*	Retention time (min)*
3,4-dihydroxibenzoic acid	260	4.23
(+)-catechin	280	6.68
Myricetin 3-glucoside	260	15.88
Rutine	260	20.02
Quercertin 3-glucoside	260	21.37
Kaempferol 3-glucoside	260	26.05
Myricetin	370	28.51
Quercetin	370	39.32
Kaempferol	370	47.89

* The values presented here are obtained experimentally. You may obtain slightly different values due to variations in HPLC equipment, reagents, solvents, among others.

13 References

Albuquerque B., Heleno S., Oliveira B., Barros L., Ferreira I. (2021). Phenolic compounds: current industrial applications, limitations and future challenges. Food & Function, 12, 14–29. https://doi.org/10.1039/D0FO02324H

Alfaro-Diaz A., Escobedo A., Luna-Vital D.A., Castillo-Herrera G., Mojica, L. (2023). Common beans as a source of food ingredients: Techno-functional and biological potential. Comprehensive Reviews in Food Science and Food Safety, 22, 2910–2944. https://doi.org/10.1111/1541-4337.13166

Giusti F., Capuano E., Sagratini G.; Pellegrini N. (2019). A comprehensive investigation of the behaviour of phenolic compounds in legumes during domestic cooking and in vitro digestion. Food Chemistry, 285, 458–467. https://doi.org/10.1016/j.foodchem.2019.01.148

Lin L., Harnly J.M., Pastor-Corrales M.S., Luthria D. L. (2008). The polyphenolic profiles of common bean (Phaseolus vulgaris L.). Food Chemistry, 107(1), 399–410. https://doi.org/10.1038/jid.2014.371

Luthria D.L., Pastor-Corrales M.A. (2006). Phenolic acids content of fifteen dry edible bean (Phaseolus vulgaris L.) varieties. Journal of Food Composition and Analysis, 19(2–3), 205–211. https://doi.org/10.1016/j.jfca.2005.09.003

Orozco J.C., Taleon V., Patiño G., Gallego S. (2024). SOP for quantification of total phytates in beans, rice, and maize flour. Nutrition Quality Laboratory Standard Operating Procedure. International Center for Tropical Agriculture (CIAT), Cali, Colombia. 19 p. https://hdl.handle.net/10568/158299

Rodríguez Madrera R., Campa Negrillo A., Suárez Valles B., Ferreira Fernández J.J. (2021). Phenolic content and antioxidant activity in seeds of common Bean (Phaseolus vulgaris L.). Foods, 10, 864. https://doi.org/10.3390/foods10040864

14 History of document modifications

Effective date of the SOP	Version number	Description	Reviewed by
June 2025	001	Original SOP	Victor Taleon Sonia Gallego

15 Security elements

- Reagent Safety Sheet
- Lab coat
- Long pants
- Closed toe shoes
- Chemical resistant gloves (Nitrile gloves)
- Safety glasses
- Extraction cabin

16 Notes and suggestions

All solutions and extracts must be shaken properly to obtain homogeneity.

During the extract reconstitution stage, it is suggested to sonicate for the shortest possible time. Prolonged exposure to sonication can degrade some phenolic compounds, especially those sensitive to heat or the high temperatures generated by cavitation.

Before starting the injection sequence for each sample into the HPLC instrument, ensure that the HPLC system pressure is stable.

The examples described in this SOP are for demonstration purposes only. Analysts should be clear about how to apply these examples in their analytical scenario.

Laboratory Standard Operating Procedure **Determination and quantification of phenolic compounds in bean flour by HPLC**	Alliance Bioversity & CIAT · CGIAR Nutrition Quality Laboratory · FoodLab Processing and Product Development
General information	
Process tittle: Determination and quantification of phenolic compounds in bean flour by HPLC	
Author: Juan Camilo Orozco Agredo	**SOP ID:** NQL-2025-001
Contact Info: j.orozco@cgiar.org	**Date:** 05/16/2025
Company/Institution: Alliance Bioversity & CIAT	**Version:** 001
This document has been reviewed by:	
Victor TALEON	04/15/2025
Final validation by:	
Sonia GALLEGO	05/16/2025

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The CGIAR Breeding for Tomorrow Program aims to maximize CGIAR and partners' returns on investment in breeding, seed systems, and other Programs and Accelerators by creating a collaboration hub to develop institutional standards for inclusive and impact-driven market segmentation and product profiling and developing a global platform for sharing market intelligence and investment prioritization.

The CGIAR Market Intelligence Area of Work aims to maximize the impact and return on investment of breeding programs by integrating market insights, behavioral intelligence, and strategic prioritization. It identifies high-impact opportunities, guides product development, and enhances product adoption and lifecycle management through decision-support tools.

To learn more about the Breeding for Tomorrow program, please visit
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June 2025

Market Intelligence
Area of Work