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Title: Integrated Microplate and HPTLC Strategy for Determining and Profiling Total Phenolics and Flavonols in Citrus Peel Extracts

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Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

☒ Correct

2. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

3. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

If **Yes**, we will need you to record using screen recording software.

We recommend using the screen capture program [OBS](https://obsproject.com/). JoVE's tutorial for using OBS Studio is provided at this link: <https://review.jove.com/v/5848/screen-capture-instructions-for-authors?status=a7854k>

4. Proposed filming date: To help JoVE process and publish your video in a timely manner, please indicate the proposed date that your group will film here: **04/24/2026**

When you are ready to submit your video files, please contact our Content Manager, [Utkarsh Khare](#).

Current Protocol Length

Number of Steps: 20

Number of Shots: 53

Introduction

Authors: Please note that the questions will not appear on screen. Please answer in stand-alone full sentence with sufficient context. The answers have been revised for length, clarity, and consistency with journal style guidelines.

INTRODUCTION:

REQUIRED: Summarize your research focus and the main questions it aims to answer.

- 1.1. **Pablo Jiménez-Bonilla:** This protocol uses microplate and HPTLC analysis to measure phenolics and flavonoids in citrus peel extracts.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Describe the research contexts or systems in which this protocol can be applied.

- 1.2. **Pablo Jiménez-Bonilla:** This protocol applies to plant materials, especially citrus by-products, in food science, phytochemistry, and extract quality control.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

Describe the most important consideration or challenge when performing this protocol.

- 1.3. **Victor Álvarez-Valverde:** In this protocol, ensuring good chromatographic separation is critical; mobile phase polarity must be adjusted according to plant and tissue.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Are there any additional methods or analyzes that can be performed following this procedure?

- 1.4. **Victor Álvarez-Valverde:** Separated bands obtained from this procedure can be further elucidated by MS and NMR or compared with standards for identification.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

How can future studies build on this work? Describe the questions or research directions that can be investigated next.

- 1.5. **Victor Álvarez-Valverde:** This method can be developed to enable the identification of additional phenolic compounds, for a more comprehensive analysis.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Citrus Peel Processing and Sample Preparation

Demonstrator: Irina Saborío-González

- 2.1. To begin, obtain citrus fruits like lime or mandarin [1]. Remove approximately 50 grams of peel from each fruit and separate it from the edible portion [2]. Freeze the peels in a zip-lock bag at minus 20 degrees Celsius for 2 hours [3].
 - 2.1.1. WIDE: Show the work bench with the required number of citrus fruits on it.
 - 2.1.2. Talent removing peel from each fruit and separating it from the edible portion.
 - 2.1.3. Talent placing the peels in a plastic zip-lock bag and transferring the bag to a freezer.
- 2.2. Keep the frozen peels in a precooled freeze-dryer in open bags. [1]. Apply a vacuum of 0.1 millibar and dry the peels overnight until completely dry [2]. Use a blade mill to grind each dried peel material to approximately 0.1 millimeter [3].
 - 2.2.1. Talent transferring the frozen peel bags to a precooled freeze-dryer.
 - 2.2.2. Talent placing the open bags in the freeze-dryer.
 - 2.2.3. Show the dried peel and then show the talent grinding the dried peel material with a blade mill.

3. Preparation of Citrus Peel Extracts

- 3.1. Weigh around 100 milligrams of dry, ground citrus peel and place it in a 15-milliliter glass tube [1]. Add 3 milliliters of 60 percent ethanol to the tube [2].
 - 3.1.1. Talent weighing the dry, ground citrus peel and transferring it to a 15 mL glass tube.
 - 3.1.2. Talent adding 3 mL of 60% ethanol to the glass tube.
- 3.2. Sonicate the mixture in an ultrasonic bath for 10 minutes at 40 degrees Celsius [1]. Then, centrifuge the sample at 850 g for 10 minutes [2].
 - 3.2.1. Talent placing the glass tube in an ultrasonic bath.
 - 3.2.2. Talent placing the tube in a centrifuge.
- 3.3. Use a Pasteur pipette to transfer the supernatant to a 10-milliliter volumetric flask without disturbing the sediment [1]. Add 3 milliliters of 60 percent ethanol to the tube containing the moist solid residue [2]. Sonicate the mixture once more [3] and centrifuge it under the same conditions as demonstrated previously. [4]. NOTE: The VO

is edited for the additional shot

- 3.3.1. Talent transferring the supernatant to a 10-mL volumetric flask with a Pasteur pipette while leaving the sediment undisturbed.
 - 3.3.2. Talent adding 3 milliliters of 60 percent ethanol to the tube containing the moist residue.
 - 3.3.3. Representative shot of talent placing the tube into the ultrasonic bath
- Added shot 3.4.0: Sample is being centrifuged.

3.4. Use a Pasteur pipette to transfer the supernatant to the volumetric flask used earlier [1]. Add 3 milliliters of 60% ethanol to the remaining residue to repeat the extraction process [2]. Transfer the resulting supernatant to the same volumetric flask [3].

- 3.4.1. Talent transferring the second supernatant into the same volumetric flask.
- 3.4.2. Talent adding 3 milliliters of 60 percent ethanol to the remaining residue.
- 3.4.3. Shot of the talent transferring the supernatant to the volumetric flask.

3.5. Add 2 drops of 0.1 mole per liter hydrochloric acid to acidulate the combined extract [1] and bring the volume to 10 milliliters with methanol [2].

- 3.5.1. Talent adding 2 drops of 0.1 mole per liter hydrochloric acid to the combined extract.
- 3.5.2. Talent filling the volumetric flask to 10 milliliters with methanol.

4. Quercetin Standard Preparation and Flavonoid Quantification Assay

4.1. Prepare a quercetin stock solution in a 10-milliliter volumetric flask [1] [2] and add the required volume of stock solution into 1.5-milliliter tubes to prepare standards at 0, 10, 20, 40, 60, 80, and 100 micrograms per milliliter [3]. Then, add 80% methanol to bring the total to 1 milliliter [4].

- 4.1.1. Shot of the talent adding the reagents to the flask to prepare the stock solution.
TXT: See the text for the detailed quercetin stock preparation protocol
- 4.1.2. TEXT ON A PLAIN BACKGROUND
Quercetin: 10 mg
95% ethanol: ~5 mL
60% ethanol: For volume make-up. *Video editor: Please use split screen to show both the shots side by side*
- 4.1.3. Talent dispensing appropriate volume of stock solution into labeled 1.5 mL tubes.
- 4.1.4. Talent making up the volume in each tube to 1 mL volume using 80% methanol.

- 4.2. Dispense 60 microliters of blank, sample, or standard into each microplate well as per the layout [1]. Prepare 3 replicates for each sample or standard [2].
 - 4.2.1. Talent dispensing 60 microliters of blank, sample, or standard into the microplate wells. *Video editor: Please add LAB MEDIA - Figure 1(b) as an inset*
 - 4.2.2. Shot of the microplate showing triplicate loading for each sample and standard.
- 4.3. Keep aqueous solutions of 2 percent aluminum chloride, 5 percent sodium acetate, and 80 percent methanol ready in separate reagent reservoirs [1]. Use an 8-channel pipette to add 10 microliters of aluminum chloride solution, 10 microliters of sodium acetate solution, and 200 microliters of 80 percent methanol to each well [2].
 - 4.3.1. Shot of the reagents kept in separate reagent reservoirs.
 - 4.3.2. Shot of the talent using an 8-channel pipette to add 10 microliters of aluminum chloride solution, 10 microliters of sodium acetate solution, and 200 microliters of 80 percent methanol into each well.
- 4.4. Shake the plate at intermediate intensity for 10 seconds and incubate it in the dark for 30 minutes [1]. ~~If shaking is unavailable, mix the wells manually with a micropipette before incubation [2].~~ NOTE: The VO is edited for the deleted shot
 - 4.4.1. Talent placing the microplate on a shaker and then transferring it for dark incubation. NOTE: 5.3.3a is not in the script however match the description of 4.4 (shake 10 secs and incubate 30 min)
 - 4.4.2. ~~Talent mixing the wells manually with a micropipette.~~ NOTE: Not filmed
- 4.5. Measure absorbance at 415 nanometers [1]. Interpolate the sample absorbance values from the calibration curve and apply dilution, volumetric, and mass correction factors as needed [2].
 - 4.5.1. Talent placing the microplate into a reader for absorbance measurement at 415 nanometers.
 - 4.5.2. SCREEN: 5.3.3b.mp4

5. Gallic Acid Standard Preparation and Total Phenolic Assay

- 5.1. Prepare gallic acid stock by dissolving 0.0123 grams of gallic acid in distilled water [1]. Prepare the working solution and calibration standard solutions in distilled water using

the reagents as presented [2].

5.1.1. Shot of the talent adding reagents in clearly labeled tubes or flasks.

5.1.2. LAB MEDIA: Table 2 **TXT: Working solution: 1mL stock solution in 10 mL dH₂O**

5.2. Next, prepare the Folin - Ciocalteu working reagent by diluting the commercial reagent 1 to 14 with distilled water [1]. Pipette 30 microliters of each sample, standard, or blank into a 96-well plate according to the layout, ensuring 3 replicates per sample [2]. Use a multichannel pipette to add 215 microliters of Folin–Ciocalteu working reagent to each well [3].

5.2.1. Talent diluting the commercial Folin–Ciocalteu reagent with distilled water.

5.2.2. Shot of the talent loading 30 μ L of each sample, standard, or blank into the 96-well microplate. *Video editor: Please add LAB MEDIA - Figure 3(a) as an inset.*

5.2.3. Talent adding Folin–Ciocalteu working reagent to each well with a multichannel pipette.

5.3. Add 50 microliters of 20 percent sodium carbonate solution to each well and mix thoroughly using a micropipette [1]. After incubation at 40 degrees Celsius for 20 minutes with agitation, use a plate reader to measure the absorbance at 755 nanometers [2].

5.3.1. Talent adding 50 μ L of 20 % sodium carbonate solution to each well and mixing with a micropipette.

5.3.2. Talent placing the microplate into a reader for absorbance measurement at 755 nanometers.

6. Standard Preparation and High-Performance TLC Analysis

6.1. For standard curves, prepare stock solutions of quercetin and gallic acid at 100 micrograms per milliliter [1]. For thin-layer chromatography, or TLC (*T-L-C*), use a 10-by-20-centimeter silica gel 60 F₂₅₄ (*sixty F two fifty-four*) aluminum-backed TLC plate as the stationary phase [2]. After setting up the desired volumes in the software, apply gallic and quercetin individually to separate lanes of the plate TLC for identification [3].

NOTE: The VO is edited for the moved and modified shots

6.1.1. Shot of the clearly labelled quercetin and gallic acid stock solutions.

6.2.1 Shot of the silica gel 60 F₂₅₄ aluminum-backed thin-layer chromatography plate.

NOTE: 6.2.1 has been moved here as per authors request

- 6.1.2. Talent applying gallic acid and quercetin. **NOTE: Use 6.1.2c and 6.1.2d. The video does not show the exact shot, it just show how machine performs the task**
- 6.1.3. Talent applying gallic acid and quercetin individually to separate lanes.

~~6.2. For thin layer chromatography or TLC (TLC), use a 10 by 20 centimeter silica gel 60 F₂₅₄ (sixty F two fifty four) aluminum backed TLC plate as the stationary phase [1]. Set the application parameters to a Y position of 7.0 millimeters, a band length of 6.0 millimeters, a first X position of 14.0 millimeters, and a distance between tracks of 9.4 millimeters [2]. Apply 10 microliters of each extract per band using nitrogen as the spraying gas [3]. Set the solvent front distance to 70 millimeters [4].~~ **NOTE: not filmed**

- ~~6.2.1. Shot of the silica gel 60 F₂₅₄ aluminum backed thin layer chromatography plate.~~
- ~~6.2.2. **SCREEN:** Display the application parameter settings with Y position 7.0 millimeters, band length 6.0 millimeters, first X position 14.0 millimeters, and distance between tracks 9.4 millimeters.~~
- ~~6.2.3. **SCREEN:** 10 microliters of each extract is being applied per band using nitrogen as the spraying gas.~~
- ~~6.2.4. **SCREEN:** The solvent front distance is being set at 70 millimeters.~~

6.3. After setting the plate layout parameters, keep the solutions for the mobile phases ready [1][2]. Saturate the chamber for 10 minutes, with filter paper in contact with the mobile phase and the chamber wall [3]. Develop the plate in a manual chamber containing 40 milliliters of the appropriate mobile phase [4]. **NOTE: The VO is edited as per the inverted shots**

- 6.3.1. Talent keeping the solutions for the mobile phases ready
- 6.3.2. TEXT ON A PLAIN BACKGROUND:
 Mobile phase 1 (MP1):
 Ethyl acetate: Formic acid: Acetic acid: Water - 100:11:11:26
- Mobile phase 2 (MP2):
 Toluene: Ethyl acetate: Formic acid - 5:4:1 *Video editor: Please use split screen to show both the shots side by side*
- 6.3.4. Talent placing filter paper in contact with the mobile phase and the chamber wall for saturation. **NOTE: 6.3.3. and 6.3.4 has been inverted**
- 6.3.3. Talent placing the thin-layer chromatography plate into a manual chamber containing 40 milliliters of mobile phase. **NOTE: 6.3.3" shot is showing both 6.3.3 and 6.3.4**

7. TLC Plate Development

- 7.1. Apply the samples in triplicate lanes together with the gallic acid and quercetin reference lanes for identification [1]. Develop the plate using mobile phase 1 [2] and then again using mobile phase 2 [3].
 - 7.1.1. Talent applying samples in triplicate lanes together with the reference lanes.
 - 7.1.2. Talent placing the plate into the chamber for development with MP1.
 - 7.1.3. Talent placing the plate into the chamber for development with MP2.
- 7.2. After derivatizing the plate with Folin–Ciocalteu reagent, photo-document the plate under white light and ultraviolet illumination at 254 and 366 nanometers [1]. Use a separate TLC plate to repeat the procedure, derivatize it with aluminum chloride, and photo-document it as demonstrated previously [2].
 - 7.2.1. Talent photo-documenting the plate under white illumination and under 254 nanometer and 366 nanometer ultraviolet illumination.
 - 7.2.2. Talent repeating the procedure.

Results

8. Results

8.1. After aluminum chloride derivatization, the lime extract showed 2 bands at Rf 0.46 [1] and Rf 0.62 [2], while no corresponding bands were detected in mandarin [3].

8.1.1. LAB MEDIA: Figure 5. *Video editor: Highlight the band in the lime lane under "AlCl₃-366" at Rf 0.46 (a little below 0.5)*

8.1.2. LAB MEDIA: Figure 5. *Video editor: Highlight the band in the lime lane under "AlCl₃-366" at Rf 0.62 (a little above 0.6)*

8.1.3. LAB MEDIA: Figure 5. *Video editor: Show the mandarin lane under "AlCl₃-366" and indicate the absence of bands at the above-mentioned positions*

8.2. The more polar mobile phase 1 provided superior separation of phenolic constituents compared to mobile phase 2 [1], while the less polar mobile phase 2 enabled the simultaneous retention of both sample components and reference standards within the same chromatographic run [2].

8.2.1. LAB MEDIA: Figure 6A. *Video editor: Show panel A and emphasize that the bands are well spread out across the plate.*

8.2.2. LAB MEDIA: Figure 6B. *Video editor: Show panel B and highlight that lime, mandarin, gallic acid, and quercetin tracks are all present together on the plate.*

8.3. In both lime and mandarin extracts, 2 distinct bands were observed [1] in addition to a band remaining at the origin [2], and a yellow band with a retention factor value of 0.14 became clearly visible after aluminum chloride derivatization [3].

8.3.1. LAB MEDIA: Figure 6. *Video editor: Highlight the 2 separate visible bands in the lime and mandarin tracks in the upper middle panel*

8.3.2. LAB MEDIA: Figure 6. *Video editor: Zoom in on the bottom of the lime and mandarin tracks to show the band that stayed at the origin.*

8.3.3. LAB MEDIA: Figure 6. *Video editor: Highlight the yellow band in AlCl₃ panels*

1. Citrus

Pronunciation link: <https://www.merriam-webster.com/dictionary/citrus>

- IPA: /'sɪ.trəs/
Phonetic Spelling: sit·ruhs
2. Mandarin
Pronunciation link: <https://www.merriam-webster.com/dictionary/mandarin>
IPA: /'mæn.də.ɪn/
Phonetic Spelling: man·duh·rin
 3. Freeze-dryer
Pronunciation link: <https://www.merriam-webster.com/dictionary/freeze-dry>
IPA: /'fri:z.draɪ.ə/
Phonetic Spelling: freez·dry·er
 4. Sonicate
Pronunciation link: <https://www.merriam-webster.com/dictionary/sonicate>
IPA: /'sɑ:.nə.keɪt/
Phonetic Spelling: son·uh·kayt
 5. Ultrasonic
Pronunciation link: <https://www.merriam-webster.com/dictionary/ultrasonic>
IPA: /,ʌl.trə'sɑ:.nɪk/
Phonetic Spelling: uhl·truh·son·ik
 6. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /'sen.trə.fju:dʒ/
Phonetic Spelling: sen·truh·fyooj
 7. Pasteur pipette
Pronunciation link: No confirmed link found
IPA: /pæ'stɜ:.paɪ'pet/
Phonetic Spelling: pas·tur pie·pet
 8. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /,su:.pə'nei.tənt/
Phonetic Spelling: soo·per·nay·tuht
 9. Volumetric
Pronunciation link: <https://www.merriam-webster.com/dictionary/volumetric>
IPA: /,vɑ:.ljə'me.trɪk/
Phonetic Spelling: vol·yuh·met·rik
 10. Hydrochloric acid
Pronunciation link: <https://www.merriam-webster.com/dictionary/hydrochloric%20acid>
IPA: /,haɪ.droʊ'klɔ:r.ɪk 'æs.ɪd/
Phonetic Spelling: hy·droh·klor·ik ass·id
 11. Methanol
Pronunciation link: <https://www.merriam-webster.com/dictionary/methanol>
IPA: /'meθ.ə.nɔ:l/
Phonetic Spelling: meth·uh·nawl
 12. Quercetin
Pronunciation link: <https://www.merriam-webster.com/dictionary/quercetin>

- IPA: /'kwɜː.sə.tɪn/
Phonetic Spelling: kwer·suh·tin
13. Flavonoid
Pronunciation link: <https://www.merriam-webster.com/dictionary/flavonoid>
IPA: /'fleɪ.vəˌnɔɪd/
Phonetic Spelling: flay·vuh·noyd
14. Aqueous
Pronunciation link: <https://www.merriam-webster.com/dictionary/aqueous>
IPA: /'eɪ.kwi.əs/
Phonetic Spelling: ay·kwee·uhs
15. Aluminum chloride
Pronunciation link: No confirmed link found
IPA: /ə'luː.mən.nəm 'klɔːr.aɪd/
Phonetic Spelling: uh·loo·muh·nuhm klor·ide
16. Sodium acetate
Pronunciation link: No confirmed link found
IPA: /'soʊ.di.əm 'æs.əˌteɪt/
Phonetic Spelling: soh·dee·uhm ass·uh·tayt
17. Absorbance
Pronunciation link: <https://www.merriam-webster.com/dictionary/absorbance>
IPA: /əb'zɔːr.bəns/
Phonetic Spelling: ub·zor·buhns
18. Interpolate
Pronunciation link: <https://www.merriam-webster.com/dictionary/interpolate>
IPA: /ɪn'tɜː.pəˌleɪt/
Phonetic Spelling: in·ter·puh·layt
19. Gallic acid
Pronunciation link: <https://www.merriam-webster.com/dictionary/gallic%20acid>
IPA: /'gæl.ɪk 'æs.ɪd/
Phonetic Spelling: gal·ik ass·id
20. Phenolic
Pronunciation link: <https://www.merriam-webster.com/dictionary/phenolic>
IPA: /fi'naː.lɪk/
Phonetic Spelling: fih·nol·ik
21. Folin–Ciocalteu
Pronunciation link: No confirmed link found
IPA: No verified IPA available
Phonetic Spelling: foh·lin see·oh·kal·too
22. Sodium carbonate
Pronunciation link: No confirmed link found
IPA: /'soʊ.di.əm 'kɑːr.bəˌneɪt/
Phonetic Spelling: soh·dee·uhm kar·buh·nayt
23. High-performance thin-layer chromatography
Pronunciation link: No confirmed link found

IPA: /ˌhaɪ pəˈfɔːr.məns ˌθɪnˈleɪ.ə ˌkroʊ.məˈtɑːɡ.rə.fi/

Phonetic Spelling: high per·for·muhns thin·lay·er kroh·muh·tog·ruh·fee

24. Silica gel

Pronunciation link: <https://www.merriam-webster.com/dictionary/silica%20gel>

IPA: /ˈsɪl.ɪ.kə ˈdʒel/

Phonetic Spelling: sil·ih·kuh jel

25. Stationary phase

Pronunciation link: No confirmed link found

IPA: /ˈsteɪ.ʃəˌner.i feɪz/

Phonetic Spelling: stay·shuh·nair·ee fayz

26. Ethyl acetate

Pronunciation link: No confirmed link found

IPA: /ˈeθ.əl ˈæs.əˌteɪt/

Phonetic Spelling: eth·uhl ass·uh·tayt

27. Formic acid

Pronunciation link: <https://www.merriam-webster.com/dictionary/formic%20acid>

IPA: /ˈfɔːr.mɪk ˈæs.ɪd/

Phonetic Spelling: for·mik ass·id

28. Acetic acid

Pronunciation link: <https://www.merriam-webster.com/dictionary/acetic%20acid>

IPA: /əˈsiː.tɪk ˈæs.ɪd/

Phonetic Spelling: uh·see·tik ass·id

29. Toluene

Pronunciation link: <https://www.merriam-webster.com/dictionary/toluene>

IPA: /ˈtɑː.ljuː.iːn/

Phonetic Spelling: tol·yoo·een

30. Derivatize

Pronunciation link: <https://www.merriam-webster.com/dictionary/derivatize>

IPA: /dəˈrɪv.əˌtaɪz/

Phonetic Spelling: duh·riv·uh·tyze

31. Ultraviolet

Pronunciation link: <https://www.merriam-webster.com/dictionary/ultraviolet>

IPA: /ˌʌl.trəˈvaɪ.ə.lət/

Phonetic Spelling: uhl·truh·vy·uh·luht

32. Chromatographic

Pronunciation link: <https://www.merriam-webster.com/dictionary/chromatographic>

IPA: /ˌkroʊ.mə.təˈɡræf.ɪk/

Phonetic Spelling: kroh·muh·tuh·graf·ik