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Title: Flavonoid Content During the Growth and Floral Development of *Calendula officinalis* L.

Authors and Affiliations:

Mariana Palma-Tenango[1,2], Marcos Soto-Hernández[3], Rubén San Miguel-Chávez[3], Araceli Gaytán- Acuña[4], Víctor A. González- Hernández[1]

[1]Posgrado en Fisiología Vegetal. Recursos Genéticos y Productividad, Colegio de Postgraduados-Campus Montecillo
[2]Facultad de Ciencias, Universidad Nacional Autónoma de México, Ciudad Universitaria
[3]Posgrado en Botánica, Colegio de Postgraduados-Campus Montecillo
[4]Posgrado en Fruticultura, Colegio de Postgraduados-Campus Montecillo

Corresponding Authors:

Marcos Soto-Hernandez (msoto@colpos.mx)

Email Addresses for All Authors:

Mariana Palma-Tenango (marianapt@ciencias.unam.mx)
Marcos Soto-Hernandez (msoto@colpos.mx)
Rubén San Miguel-Chávez (sanmi@colpos.mx)
Araceli Gaytán- Acuña (egaytan@colpos.mx)
Víctor A. González- Hernández (vagh@colpos.mx)

Author Questionnaire

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

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **NO**

3. **Filming location:** Will the filming need to take place in multiple locations? **Yes**

   How far apart are the locations? **10 meters**

Current Protocol Length
Number of Steps: 14
Number of Shots: 23

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

REQUIRED:

1.1. **Marcos Soto-Hernandez:** Our study uses an accessible and reliable colorimetric method to quantify total flavonoids, facilitating their analysis in phytochemistry laboratories and supporting the research of compounds with therapeutic properties.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 5.*

What are the most recent developments in your field of research?

1.2. **Rubén San Miguel-Chávez :** The flavonoid content in *Calendula* is studied during floral development to identify the optimal harvest time and maximize its medicinal and commercial potential.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Table 2.*

What advantage does your protocol offer compared to other techniques?

1.3. **Mariana Palma-Tenango:** Our protocol is a sensitive and efficient microtechnique that enables the quantification of flavonoids using small sample amounts, optimizing solvent use and facilitating analysis when plant material is limited.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.7.1., 2.7.2.*

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions:

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE?

1.4. **Rubén San Miguel-Chávez:** By publishing in JoVE, we hope to strengthen collaboration with other research groups, reduce laboratory supply costs, and improve overall productivity.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Quantification of Total Flavonoids in *Calendula officinalis* L. Flowerheads

Demonstrators: Mariana Palma-Tenango and Rubén San Miguel-Chávez

2.1. To begin, dry the plant material in a forced-air oven at 40 degrees Celsius for 48 hours **[1]**. Once dry, remove it from the oven **[2]**. NOTE: The VO has been edited.

2.1.1. WIDE: Talent placing the plant material inside the forced-air oven and setting the temperature to 40 degrees Celsius.

Added shot: Check that the plant material is dry and remove it from the oven.

2.2. Transfer the dried material into paper envelopes **[1]** and store them in the dark at room temperature **[2]**.

2.2.1. Talent transferring the dried plant material into paper envelopes.

2.2.2. Talent placing the envelopes in the dark at room temperature.

2.3. Freeze the plant material using liquid nitrogen to prepare for grinding **[1]**.

2.3.1. Talent pouring liquid nitrogen onto the plant material to freeze it.

2.4. Then, grind the frozen material in a porcelain mortar until it reaches a uniform texture **[1]**.

2.4.1. Talent using a porcelain mortar and pestle to grind the frozen plant material to a fine, uniform texture.

2.5. Prepare the samples by weighing 25 milligrams of the pulverized material from each sample **[1]**. Add 500 microliters of 80 percent methanol to the weighed material **[2]**.

2.5.1. Talent weighing 25 milligrams of pulverized plant material on a balance.

2.5.2. Talent adding 500 microliters of 80 percent methanol to the sample using a

micropipette.

2.6. To extract flavonoids, incubate the mixture at 70 degrees Celsius for 1 hour **[1]**. After incubation, centrifuge the mixture at 731 *g* for 13 minutes **[2]**.

2.6.1. Talent placing the sample tubes into an incubator.

2.6.2. Talent placing the tubes into a centrifuge.

2.7. Now, prepare aliquots by taking 150 microliters of the obtained extract **[1]** and adding 37 microliters of 80 percent methanol to it **[2]**.

2.7.1. Talent pipetting 150 microliters of extract into a new tube.

2.7.2. Talent adding 37 microliters of 80 percent methanol to the extract.

2.8. Take 50 microliters of the extract **[1]** and add 100 microliters of 1 molar potassium acetate and 100 microliters of 10 percent aluminum chloride **[2]**.

2.8.1. Talent pipetting 50 microliters of extract into a new tube.

2.8.2. Talent sequentially adding 100 microliters of 1 molar potassium acetate and 100 microliters of 10 percent aluminum chloride to the tube.

2.9. Complete the solution by bringing the total volume of the mixture up to 5 milliliters using distilled water **[1-TXT]**.

2.9.1. Talent adding distilled water to the tube until the total volume reaches 5 milliliters. **TXT: Allow the solution to rest for 30 min at RT**

2.10. Set the absorbance wavelength of a spectrophotometer to 415 nanometers **[1]** and measure the absorbance of the solution **[2]**. NOTE: The VO has been edited.

2.10.2 Talent setting the absorbance wavelength to 415 nanometers. NOTE: This shot is moved here.

2.10.1. Talent placing the sample into the spectrophotometer. NOTE: This shot is moved here. Videographer's NOTE: Shots 2.10.1 and 2.10.2 swap order, as it is done by

the research team.

2.11. Generate the calibration curve by preparing quercetin solutions ranging from 50, 100, 175, and 350 milligrams per milliliter **[1]**.

2.11.1. A shot of the series of quercetin solutions with concentrations between 50 and 350 mg/mL placed on a workbench or inside a chemical hood. *Videographer: Please make sure the labels are visible in the frame.*

2.12. To prepare calibration samples, pipette 500 microliters of the quercetin solution from the last point of the calibration curve **[1]**, and add 100 microliters of 10 percent aluminum chloride **[2]** and 100 microliters of 1 molar potassium acetate to the solution **[3].** Then, add 1.5 milliliters of 80% methanol **[4]** and 2.8 milliliters of distilled water **[5]**. NOTE: The VO has been edited.

Added shot: 2.12.0.: Talent pipetting 500 microliters from the last point of the calibration curve.

2.12.2. Talent pipetting 100 microliters of 10 percent aluminum chloride into quercetin solution. NOTE: This shot is moved here.

2.12.1. Talent pipetting 100 microliters of 1 molar potassium acetate into quercetin solution. NOTE: This shot is moved here.

Added shot: Talent pipetting 1.5 mL of methanol 80%.

Added shot: Talent pipetting 2.8 mL of distilled water.

2.13. Let the calibration solutions rest for 40 minutes at room temperature **[1]**.

2.13.1. A shot of the calibration samples placed on the bench.

2.14. Set the absorbance wavelength of the spectrophotometer to 415 nanometers **[1]** and measure the absorbance of the calibration samples **[2]** to construct the calibration curve **[3].** Finally, calculate the flavonoid concentration and flavonoid content per gram of dry material **[4]**. NOTE: The VO has been edited.

2.14.2. Talent setting the absorbance wavelength to 415 nanometers and recording the absorbance values. NOTE: This shot is moved here. Videographer's NOTE: Shots 2.14.1 and 2.14.2 swap order, as it is done by the research team.

2.14.1. Talent placing the calibration sample into the spectrophotometer. NOTE: This shot is moved here.

2.14.3. SCREEN: Quantification-of-total-flavonoids_screenshot.mp4 00:07-00:16.

Added shot: Quantification-of-total-flavonoids_screenshot.mp4 00:22-00:32.

Results

3. **Representative Results**

3.14. This table summarizes how the flavonoid concentration and flavonoid production per plant vary from the initial bud formation to full bloom and fruit development in calendula flower heads **[1]**. Higher levels of total flavonoid concentration were observed between stages eight and eleven, corresponding to buds with separated sepals to fully opened flower heads **[2]**.

3.14.1. LAB MEDIA: Table 2.

3.14.2. LAB MEDIA: Table 2. *Video Editor: Highlight the whole rows 8 to 11.*

3.15. In earlier stages, such as buds with united sepals **[1]**, and in later stages, such as senescent flower heads **[2]**, flavonoid concentrations were 22 percent to 27 percent lower **[3]** compared to fully opened flower heads **[4]**.

3.15.1. LAB MEDIA: Table 2. *Video Editor: Highlight the whole row 7.*
3.15.2. LAB MEDIA: Table 2. *Video Editor: Highlight the whole row 13.*
3.15.3. LAB MEDIA: Table 2. *Video Editor: Highlight the whole rows 7 and 13.*
3.15.4. LAB MEDIA: Table 2. *Video Editor: Highlight the whole rows 8 to 11.*

3.16. This figure shows the measurement of total flavonoid concentration in dry matter of calendula flower heads, ligulate flowers, and tubular flowers across floral development stages **[1]**. The results suggest that understanding the variation of flavonoid concentration among different floral structures and stages is useful for determining the optimal harvest time to maximize flavonoid content in calendula flower heads **[2]**.

3.16.1. LAB MEDIA: Figure 5.
3.16.2. LAB MEDIA: Figure 5.

Pronunciation Guides:

1. Porcelain

Pronunciation link:
https://www.merriam-webster.com/dictionary/porcelain
IPA: /ˈpɔːrsəlɪn/
Phonetic Spelling: por-suh-lin

2. Micropipette

Pronunciation link:
https://www.howtopronounce.com/micropipette
IPA: /ˌmaɪkroʊpaɪˈpɛt/
Phonetic Spelling: my-kroh-py-pet

3. Methanol

Pronunciation link:
https://www.merriam-webster.com/dictionary/methanol
IPA: /ˈmɛθəˌnɔːl/
Phonetic Spelling: meth-uh-nawl

4. Flavonoid

Pronunciation link:
https://www.merriam-webster.com/dictionary/flavonoid
IPA: /ˈflævəˌnɔɪd/
Phonetic Spelling: flah-vuh-noyd

5. Centrifuge

Pronunciation link:
https://www.merriam-webster.com/dictionary/centrifuge
IPA: /ˈsɛntrəˌfjuːdʒ/
Phonetic Spelling: sen-truh-fyooj

6. Quercetin

Pronunciation link:
https://www.merriam-webster.com/dictionary/quercetin
IPA: /ˈkwɜːrsɪtɪn/
Phonetic Spelling: kwur-suh-tin

7. Calendula

Pronunciation link:
https://www.merriam-webster.com/dictionary/calendula
IPA: /kəˈlɛndjʊlə/
Phonetic Spelling: kuh-len-juh-luh

8. Ligulate

Pronunciation link:
https://www.howtopronounce.com/ligulate
IPA: /ˈlɪgjʊlɪt/
Phonetic Spelling: lig-yuh-lit

9. Tubular

Pronunciation link:
https://www.merriam-webster.com/dictionary/tubular

IPA: /ˈtuːbjələr/
Phonetic Spelling: too-byuh-ler