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Scriptwriter Name: Pallavi Sharma

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Title: An Efficient and Rapid HPLC-QQQ-MS Method for the Quantitation of Tropane Alkaloids in Medicinal Plants

Authors and Affiliations:

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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? **YES, all done**

- 3. Filming location:** Will the filming need to take place in multiple locations? **NO**

- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **NO**

Current Protocol Length

Number of Steps: 24

Number of Shots: 39

Introduction

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

INTRODUCTION:

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

- 1.1. **Zhiqi Liu:** We developed an HPLC-QQQ-MS method to rapidly quantify three major tropane alkaloids in *Anisodus tanguticus*.
 - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: 3.5.2*

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Zhiqi Liu:** Conventional HPLC lacks sensitivity for trace alkaloids; this method uses highly sensitive mass spectrometry to solve this problem.
 - 1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Zhiqi Liu:** This protocol allows rapid, accurate quantification of three major tropane alkaloids.
 - 1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

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

1.4. **Zhiqi Liu:** The key challenge is ensuring instrument precision and standardized sample preparation for accurate alkaloid measurement.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: 3.6.1*

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

1.5. **Zhiqi Liu:** This method can be extended to explore and quantify other metabolites in *Anisodus tanguticus*.

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

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

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Sample Preparation for HPLC Coupled with Triple Quadrupole Mass Spectrometry

Demonstrator: Zhiqi Liu

- 2.1. To begin, accurately weigh 10.0 milligrams of hyoscyamine, anisodamine hydrobromide, and scopolamine hydrobromide reference standards [1]. Place them into a separate 10-milliliter volumetric flask [2].
 - 2.1.1. WIDE: Talent weighing 10.0 milligrams of hyoscyamine reference standard on a one-ten-thousandth analytical balance.
 - 2.1.2. Talent placing the hyoscyamine reference standard into a 10 milliliter volumetric flask.
- 2.2. Add chromatographic-grade methanol to the marked line in each volumetric flask [1]. Dissolve the standards thoroughly with ultrasonic treatment to prepare single standard stock solutions with a concentration of 1.0 milligram per milliliter [2].
 - 2.2.1. Talent adding chromatographic-grade methanol to the marked line in each volumetric flask.
 - 2.2.2. Talent placing the volumetric flasks into ultrasonic treatment to dissolve the standards.
- 2.3. Then, transfer the stock solutions to brown reagent bottles and store at 4 degrees Celsius in the dark [1].
 - 2.3.1. Talent transferring the stock solutions to brown reagent bottles.
- 2.4. To prepare a mixed standard intermediate solution of 10 micrograms per milliliter, pipette 100 microliters of each previously prepared stock solution into a 10-milliliter volumetric flask [1]. Then, add chromatographic-grade methanol to the marked line to dilute the solution [2]
 - 2.4.1. Talent pipetting 100 microliters of each previously prepared stock solution into a 10 milliliter volumetric flask.
 - 2.4.2. Talent adding chromatographic-grade methanol to the marked line in the volumetric flask.
- 2.5. Next, perform freeze-drying of fresh *Sophora flavescens* material [1]. Using a high-speed grinder, pulverize the dried material into a homogeneous powder [2].
 - 2.5.1. Talent placing fresh *Sophora flavescens* material into a freeze-drying setup.
 - 2.5.2. Talent grinding the freeze-dried material into a homogeneous powder using a

high-speed grinder.

- 2.6. Sieve the powder through a 0.25-millimeter standard test sieve [1]. Accurately weigh 20 milligrams of the sieved powder [2], transfer it into a 100-milliliter volumetric flask [3], and add chromatographic-grade methanol to nearly the marked line [4].
 - 2.6.1. Talent sieving the powder through a 0.25-millimeter standard test sieve.
 - 2.6.2. Talent weighing 20 milligrams of the sieved powder on an analytical balance.
 - 2.6.3. Talent transferring the weighed powder into a 100 milliliter volumetric flask.
 - 2.6.4. Talent adding chromatographic-grade methanol to nearly the marked line in the volumetric flask.
- 2.7. Next, perform ultrasonic extraction at room temperature for 30 minutes [1]. Then, remove the volumetric flask and allow it to cool to 25 degrees Celsius at room temperature [2]. Add chromatographic-grade methanol to the 100-milliliter marked line and vortex to mix for 2 minutes [3].
 - 2.7.1. Talent placing the volumetric flask into ultrasonic treatment for extraction.
 - 2.7.2. Talent removes the volumetric flask and keeping it on the bench.
 - 2.7.3. Talent adding chromatographic-grade methanol to the 100 milliliter marked line and vortexing the flask.
- 2.8. Then, pipette 1 milliliter of the prepared extract into a 100-milliliter volumetric flask [1]. Add methanol to the marked line, shake well to mix thoroughly, and obtain the mother solution for HPLC injection [2].
 - 2.8.1. Talent pipetting 1 milliliter of the extract into a 100 milliliter volumetric flask.
 - 2.8.2. Talent adding methanol to the marked line in the volumetric flask and shaking it.
- 2.9. For sample filtration, prepare a 1 milliliter disposable needle-free syringe and a 0.22 micrometer organic phase microporous membrane [1].
 - 2.9.1. Talent arranging a 1 milliliter disposable needle-free syringe and a 0.22 micrometer organic phase microporous membrane on the bench.
- 2.10. Aspirate 1.2 milliliters of the mother solution for injection into the syringe [1] and push the solution slowly through the membrane to perform filtration [2].
 - 2.10.1. Talent aspirating 1.2 milliliters of the mother solution into the syringe.
 - 2.10.2. Talent pushing the solution slowly through the microporous membrane for filtration.
- 2.11. Collect the filtrate into a 2-milliliter sample vial with an inner insert and seal the vial tightly with a cap [1].
 - 2.11.1. Talent collecting the filtrate into a 2 milliliter sample vial with an inner insert and

sealing the sample vial tightly with a cap.

3. HPLC–ESI–MS/MS Workflow for Quantification and Validation of Analytes

- 3.1. Prepare mobile phase A by measuring 1000 milliliters of ultrapure water [1]. Add 1.0 milliliter of chromatographic-grade formic acid [2] and mix uniformly using a magnetic stirrer for 10 minutes [3].
 - 3.1.1. WIDE: Talent measuring 1000 milliliters of ultrapure water into a container.
 - 3.1.2. Talent adding 1.0 milliliter of chromatographic-grade formic acid to the water.
 - 3.1.3. Talent placing the container on a magnetic stirrer and mixing the solution.
- 3.2. Next, prepare mobile phase B by using chromatographic-grade methanol directly without additional treatment [1].
 - 3.2.1. Talent holding a container of chromatographic-grade methanol.
- 3.3. Pour mobile phase A and B into ultrasonic degassing devices, respectively [1], and perform ultrasonic degassing at 40 kilohertz for 15 minutes [3].
 - 3.3.1. Talent pouring mobile phase A into an ultrasonic degassing device.
 - 3.3.2. Talent placing the solutions into ultrasonic degassing for degassing.
- 3.4. Transfer the degassed mobile phases into solvent bottles [1]. After sealing the bottles, connect them to the corresponding A and B solvent lines of the HPLC system [2].
 - 3.4.1. Talent transferring degassed mobile phase A into a solvent bottle.
 - 3.4.2. Talent connecting the solvent bottles to the A and B solvent lines of the high-performance liquid chromatography system.
- 3.5. Now, use a C18 column for chromatographic separation [1]. Equilibrate the column with methanol and water at a ratio of 80 to 20 for 30 minutes at a flow rate of 0.3 milliliters per minute [2]. Set the column oven temperature to 30 degrees Celsius and preheat for 10 minutes in advance [3].
 - 3.5.1. Talent installing a C18 column into the high-performance liquid chromatography system.
 - 3.5.2. Talent setting the mobile phase composition and initiating column equilibration.
 - 3.5.3. Display the column oven panel, set the temperature to 30 degrees Celsius, and start preheating
- 3.6. Then, set the flow rate to 0.60 milliliters per minute, the injection volume to 2 microliters, and the detection wavelength to 210 nanometers [1]. After that, set the gradient elution program with the presented conditions [2].
 - 3.6.1. SCREEN: 70510_screenshot_1.mkv: 00:45-00:55

- 3.6.2. SCREEN: TEXT ON A PLAIN BACKGROUND
- 0–10.0 min, 5% to 10% B
- 10.0–15.0 min, 10% to 35% B
- 15.0–20.0 min, 35% to 60% B
- 20.0–25.0 min, 60% to 95% B
- 3.7. Next, perform mass spectrometric detection using a triple quadrupole mass spectrometer equipped with an electrospray ionization source operating in positive ionization mode [1].
- 3.7.1. WIDE: Shot of the triple quadrupole mass spectrometer system.
- 3.8. Set the nebulizer pressure to 15 pounds per square inch [1], capillary voltage to 4000 volts [2], gas temperature to 300 degrees Celsius [3], and gas flow to 11 liters per minute [4]. Then set the parameters for multiple reaction monitoring ion pairs [5].
- 3.8.1. SCREEN: 70510_screenshot_1.mkv: 01:17-01:20
- 3.8.2. SCREEN: 70510_screenshot_1.mkv: 01:27-01:30
- 3.8.3. SCREEN: 70510_screenshot_1.mkv: 01:37-01:40
- 3.8.4. SCREEN: 70510_screenshot_1.mkv: 01:50-01:53 *Video editor: Please highlight the “Gas Flow” column*
- 3.8.5. SCREEN: 70510_screenshot_1.mkv: 02:15-02:27
- 3.9. To identify precursor ions, directly infuse individual standard solutions in **MS2 SCAN (M-S-Two-Scan)** mode [1].
- 3.9.1. Talent infusing individual standard solutions into the mass spectrometer in scan mode.
- 3.10. After method validation, perform data acquisition in multiple reaction monitoring mode [1]. Use the optimized parameters, including precursor ion, product ion transitions, fragmentor voltage, and collision energy for each analyte as presented here [2].
- 3.10.1. Talent starting data acquisition in multiple reaction monitoring mode.
- 3.10.2. LAB MEDIA: Table 1
- 3.11. Then, launch the qualitative analysis software. Select **File** and choose **Import Data** to import all collected data files [1].
- 3.11.1. SCREEN: 70510_screenshot_2.mkv: 00:00-00:13
- 3.12. Check the peak symmetry and retention time consistency of the target peaks in each extracted ion chromatogram [1].
- 3.12.1. SCREEN: 70510_screenshot_2.mkv: 00:24-00:40

3.13. In the Quantitation module, select **MRM Transition** (*M-R-M-Transition*) [1]. Input the precursor ion and product ion pairs for each component and extract the corresponding extracted ion chromatograms [2]. Calculate the peak area for quantitative analysis [3].

3.13.1. SCREEN: 70510_screenshot_2.mkv:00:44-00:55

3.13.2. SCREEN: 70510_screenshot_2.mkv: 01:10-01:35

3.13.3. SCREEN: 70510_screenshot_2.mkv: 01:56-02:04

Results

4. Results

- 4.1. All three target tropane alkaloids were successfully separated within 20 minutes under the optimized HPLC conditions [1].
 - 4.1.1. LAB MEDIA: Figure 3. *Video editor: Show the chromatogram with the three distinct peaks appearing before the 20-minute mark*
- 4.2. The resolution between scopolamine and anisodamine, and between anisodamine and hyoscyamine, both exceed the baseline separation criterion [1].
 - 4.2.1. LAB MEDIA: Figure 4. *Video editor: Highlight the separation between peak 1 and peak 2, then between peak 2 and peak 3*
- 4.3. All analytes showed good linearity over a concentration range of 0.014 to 0.320 microgram per milliliter with correlation coefficients greater than 0.99 [1].
 - 4.3.1. LAB MEDIA: Figure 5 and Table 2. *Video editor: Highlight the linear range 0.014–0.224 for scopolamine and anisodamine and 0.020–0.320 for hyoscyamine*
- 4.4. Mean recoveries were 101.44 percent for scopolamine [1], 100.25 percent for anisodamine [2], and 99.56 percent for hyoscyamine [3], with relative standard deviations of 1.85 percent, 2.35 percent, and 1.90 percent, respectively [4].
 - 4.4.1. LAB MEDIA: Table 3. *Video editor: Highlight the mean recovery value 101.44 percent and RSD of 1.84 for scopolamine*
 - 4.4.2. LAB MEDIA: Table 3. *Video editor: Highlight the mean recovery value 100.25 percent and RSD of 2.35 for anisodamine*
 - 4.4.3. LAB MEDIA: Table 3. *Video editor: Highlight the mean recovery value 99.56 percent and RSD of 1.90 for hyoscyamine*
 - 4.4.4. LAB MEDIA: Table 3. *Video editor: Highlight the RSD column*
- 4.5. Quantification of three tropane alkaloids in 10 *Anisodus tanguticus* samples showed that [1], scopolamine content ranged from 0.3667 milligram per gram in Sample 6 to 1.0356 milligram per gram in Sample 7 [2]. Anisodamine content ranged from 0.0269 milligram per gram in Sample 6 to 0.3590 milligram per gram in Sample 10 [3].
 - 4.5.1. LAB MEDIA: Table 4.
 - 4.5.2. LAB MEDIA: Table 4. *Video editor: Highlight the value 0.3667 in Sample 6 and*

1.0356 in Sample 7 under scopolamine

4.5.3. LAB MEDIA: Table 4. *Video editor: Highlight the value 0.0269 in Sample 6 0.3590 in Sample 10 under anisodamine*

4.6. Hyoscyamine content ranged from 0.2930 milligram per gram in Sample 2 [1] to 1.4989 milligram per gram in Sample 10, with higher levels observed in Samples 4, 7, 9, and 10 [2].

4.6.1. LAB MEDIA: Table 4. *Video editor: Highlight the value 0.2930 in Sample 2 under hyoscyamine*

4.6.2. LAB MEDIA: Table 4. *Video editor: Highlight the value 1.4989 in Sample 10 under hyoscyamine*

1. Hyoscyamine
Pronunciation link: <https://www.merriam-webster.com/dictionary/hyoscyamine>
IPA: /ˌhaɪ.ooˈsaɪ.ə.miːn/
Phonetic Spelling: hy·oh·sy·uh·meen
2. Anisodamine
Pronunciation link: <https://www.howtopronounce.com/anisodamine>
IPA: /əˌniːsoʊˈdæmiːn/
Phonetic Spelling: uh·nee·soh·da·meen
3. Scopolamine
Pronunciation link: <https://www.merriam-webster.com/dictionary/scopolamine>
IPA: /skoʊˈpɑːləmiːn/
Phonetic Spelling: skoh·paa·luh·meen
4. Hydrobromide
Pronunciation link: <https://www.merriam-webster.com/dictionary/hydrobromide>
IPA: /ˌhaɪdrouˈbroʊmaɪd/
Phonetic Spelling: hy·droh·broh·myde
5. Volumetric
Pronunciation link: <https://www.merriam-webster.com/dictionary/volumetric>
IPA: /ˌvɒːljəˈmɛtrɪk/
Phonetic Spelling: vol·yuh·meh·trik
6. Chromatographic
Pronunciation link: <https://www.merriam-webster.com/dictionary/chromatographic>
IPA: /ˌkroʊməˈtægrəˈfɪk/
Phonetic Spelling: kroh·muh·tag·ruh·fik
7. Ultrasonic
Pronunciation link: <https://www.merriam-webster.com/dictionary/ultrasonic>
IPA: /ˌʌltrəˈsɑːnɪk/
Phonetic Spelling: uhl·truh·son·ik
8. Sophora
Pronunciation link: <https://www.howtopronounce.com/sophora>

- IPA: /soʊˈfɔːrə/
Phonetic Spelling: soh·for·uh
9. Flavescens
Pronunciation link: <https://www.howtopronounce.com/flavescens>
IPA: /fləˈvesɛnz/
Phonetic Spelling: פֿלֶה·ves·enz
10. Homogeneous
Pronunciation link: <https://www.merriam-webster.com/dictionary/homogeneous>
IPA: /ˌhoʊməˈdʒiːniəs/
Phonetic Spelling: hoh·muh·jee·nee·uhs
11. Microporous
Pronunciation link: <https://www.merriam-webster.com/dictionary/microporous>
IPA: /ˌmaɪkroʊˈpɔːrəs/
Phonetic Spelling: my·kroh·por·uhs
12. Filtrate
Pronunciation link: <https://www.merriam-webster.com/dictionary/filtrate>
IPA: /ˈfɪltreɪt/
Phonetic Spelling: פֿיל·trayt
13. Ultrapure
Pronunciation link: <https://www.merriam-webster.com/dictionary/ultrapure>
IPA: /ˌʌltrəˈpjʊr/
Phonetic Spelling: uhl·truh·pyur
14. Formic
Pronunciation link: <https://www.merriam-webster.com/dictionary/formic>
IPA: /ˈfɔːrmɪk/
Phonetic Spelling: for·mik
15. Degassing
Pronunciation link: <https://www.merriam-webster.com/dictionary/degas>
IPA: /diːˈɡæsɪŋ/
Phonetic Spelling: dee·gas·ing
16. Chromatography
Pronunciation link: <https://www.merriam-webster.com/dictionary/chromatography>
IPA: /ˌkroʊməˈtɑːɡrəfi/
Phonetic Spelling: kroh·muh·tog·ruh·fee
17. Equilibrate
Pronunciation link: <https://www.merriam-webster.com/dictionary/equilibrate>
IPA: /ˌiːkwəˈlɪbreɪt/
Phonetic Spelling: ee·kwuh·li·brayt
18. Spectrometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/spectrometer>
IPA: /spekˈtrɑːmɪtər/
Phonetic Spelling: spek·trom·uh·ter
19. Electrospray
Pronunciation link: <https://www.merriam-webster.com/dictionary/electrospray>

IPA: /ɪˈlektroʊˌspreɪ/

Phonetic Spelling: ih·lek·troh·spray

20. Nebulizer

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

IPA: /ˈnɛbjələˌzaɪzər/

Phonetic Spelling: neb·yuh·lai·zer

21. Precursor

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

IPA: /priːˈkɜːsər/

Phonetic Spelling: pree·kur·ser

22. Chromatogram

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

IPA: /ˈkroʊmətəˌɡræm/

Phonetic Spelling: kroh·muh·tuh·gram

23. Quantitation

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

IPA: /ˌkwɑːntəˈteɪʃən/

Phonetic Spelling: kwon·tuh·tay·shun

24. Analyte

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

IPA: /ˈænəlaɪt/

Phonetic Spelling: an·uh·lite