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Project Page Link: https://review.jove.com/files_upload.php?src=21555008

Title: Technical Approach for Structural Analysis of an Unknown Compound in Huoxiang Zhengqi Oral Liquid based on Linear Ion Trap Mass Spectrometry

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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**

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: **04/20/2026**

Current Protocol Length

Number of Steps: 22

Number of Shots: 55

Interviews

NOTE to VO: Please record introductory and conclusion interview statements.

INTRODUCTION:

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

- 1.1. Chunyan Zhou: A multi-stage mass spectrometry fragmentation method was developed to identify and characterize complex constituents in Huoxiang Zhengqi oral liquid.

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

- 1.2. Chunyan Zhou: Traditional tandem mass spectrometry cannot resolve unknown compound structures; multi-stage mass spectrometry provides deeper fragmentation for comprehensive structural elucidation

CONCLUSION:

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

- 1.3. Chunyan Zhou: This protocol enables detailed analysis and structural characterization of more unknown compounds in medicinal herbs and Chinese patent medicines.

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

- 1.4. Chunyan Zhou: A clear understanding of the core structure of the main components is essential for accurate fragmentation and analysis using this protocol.

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

- 1.5. Chunyan Zhou: This procedure enables the development of a multi-stage mass spectrometry fragmentation database for improved compound identification and comparative analysis.

Protocol

NOTE: SCREEN timestamps for protocol were added at the postshoot stage. Please contact the postshoot note integrator (Sri Lakshmi Priya) for queries regarding lab media.

2. Sample Pretreatment and Ultra-Performance Liquid Chromatography Preparation

Demonstrator: Chunyan Zhou

- 2.1. To begin, obtain the commercial Huoxiang Zhengqi oral liquid or HXZQ (*H-X-Z-Q*) [1]. Transfer 0.1 milliliters of HXZQ into a 2-milliliter sample bottle [2]. Add 0.9 milliliters of water into the same bottle [3]. Shake the solution until it is thoroughly mixed [4].
 - 2.1.1. WIDE: Talent opening the packaging of the commercial HXZQ.
 - 2.1.2. Talent transferring 0.1 mL of HXZQ into a 2-mL sample bottle.
 - 2.1.3. Talent adding 0.9 mL of water into the sample bottle.
 - 2.1.4. Talent shaking the sample bottle to mix the solution thoroughly.
- 2.2. Prepare a 1 milliliter injection syringe and a 0.22 micrometer microporous membrane filter [1]. Filter the solution into a new 2-milliliter sample bottle [2].
 - 2.2.1. Talent arranging a 1 mL injection syringe and a 0.22 μ m microporous membrane filter.
 - 2.2.2. Talent filtering the solution into a new 2 mL sample bottle.
- 2.3. For ultra-performance liquid chromatography preparation, start by double-clicking on the **Xcalibur** (*X-calibur*) software to open it [1]. Click **Ready to Download**, then click **Direct Control** [2]. In the pop-up window, click the **Pump Module** column and set percentage B to 50, percentage C to 0, and percentage D to 0 [3].
 - 2.3.1. SCREEN: 70672_screenshot_2.3.1.MOV: 00:00-00:11
 - 2.3.2. SCREEN: 70672_screenshot_2.3.2.MOV: 00:05-00:14
 - 2.3.3. SCREEN: 70672_screenshot_2.3.3.MOV: 00:00-00:22
- 2.4. Click the **Motor** button to switch it to the On state [1]. Click **More Options**, set the flow to 5 milliliters per minute and the time to 180 seconds in the pop-up window [2]. Click **Purge**, then click **OK Execute Despite Warning** in the pop-up window [3].
 - 2.4.1. SCREEN: 70672_screenshot_2.4.1.MOV: 00:00-00:10
 - 2.4.2. SCREEN: 70672_screenshot_2.4.2.MOV: 00:00-00:17
 - 2.4.3. SCREEN: 70672_screenshot_2.4.3.MOV: 00:00-00:12

3. Mass Spectrometry (MS) Preparation and Full Scan MS Test

- 3.1. Return to the main software window and click **Sequence Setup View [1]**. Click **Open** to import the edited template **[2]**. Right-click on **Method Name** and click **Open File** to open the method file **[3]**.

3.1.1. SCREEN: 70672_screenshot_3.1.1.MOV: 00:00-00:10

3.1.2. SCREEN: 70672_screenshot_3.1.2.MOV: 00:00-00:11

3.1.3. SCREEN: 70672_screenshot_3.1.3.MOV: 00:00-00:15

- 3.2. In the software main window, set the **First mass** to 100 mass-to-charge ratio and the **Last mass** to 1200 mass-to-charge ratio **[1]**. Click **Save** to save the method **[2]**.

3.2.1. SCREEN: 70672_screenshot_3.2.1.MOV: 00:00-00:15

3.2.2. SCREEN: 70672_screenshot_3.2.2.MOV: 00:00-00:11

- 3.3. Click **Run sequence**, select **Standby** in the After Sequence Set System, then click **OK** in the pop-up window **[1]**. Wait for the sample injection to be completed **[2]**.

3.3.1. SCREEN: 70672_screenshot_3.3.1.MOV: 00:00-00:12

3.3.2. Shot of the talent waiting for the sample injection to be completed.

- 3.4. Click **Roadmap View**, then click the **Qual Browser** icon to open the Qual Browser window **[1]**. Click **Open**, select the data file with raw format, and double-click to open it **[2]**.

3.4.1. SCREEN: 70672_screenshot_3.4.1.MOV: 00:00-00:12

3.4.2. SCREEN: 70672_screenshot_3.4.2.MOV: 00:00-00:12

- 3.5. Right-click on the chromatogram window and click **Ranges [1]**. In the scan filter section, select **ESI Full MS (E-S-I-full-M-S)**, and in the plot type section, choose **TIC (T-I-C)**, then click **OK [2]**. Observe the total ion chromatogram displayed **[3]**.

3.5.1. SCREEN: 70672_screenshot_3.5.1.MOV: 00:00-00:08

3.5.2. SCREEN: 70672_screenshot_3.5.2.MOV: 00:00-00:15

3.5.3. SCREEN: 70672_screenshot_3.5.3.MOV: 00:00-00:08

- 3.6. Click the **Pushpin** button in the mass spectrum window **[1]**. In the chromatogram window, click and slide to select a time region with the strongest relative abundance **[2]**. Observe the corresponding mass spectrum ions and record the mass-to-charge ratio

values [3].

3.6.1. SCREEN: 70672_screenshot_3.6.1.MOV: 00:00-00:06

3.6.2. SCREEN: 70672_screenshot_3.6.2.MOV: 00:00-00:07

3.6.3. SCREEN: 70672_screenshot_3.6.3.MOV: 00:00-00:10

4. MS/MS (Tandem Mass Spectrometry)

- 4.1. Open the Instrument Setup window [1]. Locate the parent mass column of the n equals 2 row and enter the previously recorded mass-to-charge ratio value [2]. Click **Save** to save the method [3].

4.1.1. SCREEN: 70672_screenshot_4.1.1.MOV: 00:00-00:08

4.1.2. SCREEN: 70672_screenshot_4.1.2.MOV: 00:00-00:08

4.1.3. SCREEN: 70672_screenshot_4.1.3.MOV: 00:00-00:06

- 4.2. ~~Return to the software window and click **Sequence Setup View**~~ Return to the **Sequence Setup View** in the software window [1]. Modify the file name and click **Save** to save the sequence [2]. **NOTE: The VO is modified as per the shot.**

4.2.1. SCREEN: 70672_screenshot_4.2.1.MOV: 00:00-00:10

4.2.2. SCREEN: 70672_screenshot_4.2.2.MOV: 00:00-00:14

- 4.3. Click **Run sequence**, then click **OK** in the pop-up window [1]. Wait for the sample injection to be completed [2].

4.3.1. SCREEN: 70672_screenshot_4.3.1.MOV: 00:00-00:09

4.3.2. Talent observing the system during sample injection.

- 4.4. Go to the Qual Browser window, click **Open**, select the raw data file, and double-click to open it [1]. Right-click on the chromatogram window and click **Ranges** [2]. In the Scan filter section, select **ESI Full MS** [3]. In the Plot type section, choose **TIC** and click **OK** [4] to display the chromatogram [5].

4.4.1. SCREEN: 70672_screenshot_4.4.1.MOV: 00:00-00:18

4.4.2. SCREEN: 70672_screenshot_4.4.2.MOV: 00:00-00:06

4.4.3. SCREEN: 70672_screenshot_4.4.3.MOV: 00:00-00:07

4.4.4. SCREEN: 70672_screenshot_4.4.4.MOV: 00:00-00:09

4.4.5. SCREEN: 70672_screenshot_4.4.5.MOV: 00:00-00:07

- 4.5. In the mass spectrum window, click the **Pushpin (Push-pin)** button [1]. Select a time region with the strongest relative abundance and observe the mass spectrum ions [2]. Record the mass-to-charge ratio values for the next level of mass spectrometry [3].

4.5.1. SCREEN: 70672_screenshot_4.5.1.MOV: 00:00-00:06

4.5.2. SCREEN: 70672_screenshot_4.5.2.MOV: 00:00-00:07

4.5.3. SCREEN: 70672_screenshot_4.5.3.MOV: 00:00-00:06

5. Multistage Mass Spectrometry Test and Parameter Optimization

- 5.1. In the Instrument Setup window, locate the parent mass column of the n equals 3 row and enter the previously recorded mass-to-charge ratio value [1]. Click **Save** to save the method [2].

5.1.1. SCREEN: 70672_screenshot_5.1.1.MOV: 00:00-00:16

5.1.2. SCREEN: 70672_screenshot_5.1.2.MOV: 00:00-00:08

- 5.2. As previously demonstrated, repeat the data viewing procedure to complete the sample injection and analysis [1]. ~~Stop the analysis once no stable fragment ions are observed~~ After opening the raw data file, click the **Pushpin** button in the mass spectrum window and observe the changing fragment ion peaks [2]. **NOTE: The VO is modified as per the shot.**

5.2.1. *Reuse 4.2.1*

5.2.2. SCREEN: 70672_screenshot_5.2.2.MOV: 00:00-00:19

- 5.3. In the Instrument Setup window, go to the act type column and click **CID (C-I-D)** [1], then select **PQD (P-Q-D)** or **ETD (E-T-D)** to change the collision mode [2].

5.3.1. SCREEN: 70672_screenshot_5.3.1.MOV: 00:00-00:12

5.3.2. SCREEN: 70672_screenshot_5.3.2.MOV: 00:00-00:10

- 5.4. In the normalized collision energy column, click 35 and change it to 50 to adjust the collision energy [1].

5.4.1. SCREEN: 70672_screenshot_5.4.1.MOV: 00:00-00:32

6. Annotation of Parent and Fragment Ions and Structural Elucidation

- 6.1. Draw the parent ion and fragmentation ions in drawing software, including the parent ion structure, compound name, and mass-to-charge ratio value [1].

6.1.1. SCREEN: 70672_screenshot_6.1.1.MOV: 00:00-00:39

- 6.2. As an example, identify the fragment at mass-to-charge ratio 461.15 and examine the precursor ion with mass-to-charge ratio 623.21 in the tandem mass spectrometry spectrum [1]. Calculate the mass difference [2].

6.2.1. SCREEN: 70672_screenshot_6.2.1.MOV: 00:00-01:46

6.2.2. SCREEN: 70672_screenshot_6.2.2.MOV: 00:00-00:23

- 6.3. Analyze the further fragmentation of the intermediate ion with mass-to-charge ratio 461.15 to generate a product ion with mass-to-charge ratio 315.09 in the MS³ (*M-S-cubed*) [1]. Calculate the mass difference [2]. Based on the bonding position and linkage analyses of all the fragments, deduce the final structure of the unknown compound with a mass-to-charge ratio of 623.21 [3].

6.3.1. SCREEN: 70672_screenshot_6.3.1.MOV: 00:00-00:14

6.3.2. SCREEN: 70672_screenshot_6.3.2.MOV: 00:00-00:27

6.3.3. SCREEN: 70672_screenshot_6.3.3.MOV: 00:00-00:18

Results

7. Results

- 7.1. The unknown compound with a mass-to-charge ratio of 623.21 lost 1 hexose unit and yielded a fragment ion with a mass-to-charge ratio of 461.15 [1].
 - 7.1.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the first structure labelled "Unknown compound $m/z = 623.21$ " and then move to the next structure labelled "Fragment ion $m/z = 461.15$ "*
- 7.2. Tertiary mass spectrometry fragmentation of the intermediate produced neobyakangelicol with a mass-to-charge ratio of 315.09 after the loss of 1 rhamnose unit [1].
 - 7.2.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the structure labelled "Fragment ion $m/z = 461.15$ " and then move to the structure labelled "Neobyakangelicol $m/z = 315.09$ "*
- 7.3. Quadrupole mass spectrometry of neobyakangelicol produced a fragment ion with a mass-to-charge ratio of 135.09 [1].
 - 7.3.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the structure labelled "Neobyakangelicol $m/z = 315.09$ " and then move to the final structure labelled "Fragment ion $m/z = 135.09$ "*
- 7.4. The structure of the unknown compound was deduced from the multi-stage fragmentation pattern [1].
 - 7.4.1. LAB MEDIA: Figure 3A. *Video editor: Show the full left-to-right sequence of structures and arrows in panel A starting from "Unknown compound $m/z = 623.21$ " and ending at "Fragment ion $m/z = 135.09$ "*
- 7.5. The unknown compounds with mass-to-charge ratios of 545.41 [1] and 365.12 were deduced using the same fragmentation method [2].
 - 7.5.1. LAB MEDIA: Figure 4A. *Video editor: Highlight the structure labelled " $m/z = 545.41$ "*
 - 7.5.2. LAB MEDIA: Figure 4B. *Video editor: Highlight the structure labelled " $m/z = 365.12$ "*

Pronunciation guides:

1. Ultra-Performance
Pronunciation link: <https://www.merriam-webster.com/dictionary/ultraperformance>
IPA: /ˌʊl.trə pəˈfɔːr.məns/
Phonetic Spelling: ul·truh per·for·muhns
2. Chromatography
Pronunciation link: <https://www.merriam-webster.com/dictionary/chromatography>
IPA: /ˌkroʊ.məˈtɑː.grə.fi/
Phonetic Spelling: kroh·muh·taa·gruh·fee
3. Huoxiang Zhengqi
Pronunciation link: <https://www.howtopronounce.com/huoxiang-zhengqi>
IPA: /ˈhwɔː ʃjɑːŋ dʒʌŋˈtʃiː/
Phonetic Spelling: hwaw·shyaang juhng·chee
4. Micrometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>
IPA: /maɪˈkroʊ.mə.tər/
Phonetic Spelling: my·kraa·muh·tur
5. Microporous
Pronunciation link: <https://www.merriam-webster.com/dictionary/microporous>
IPA: /ˌmaɪ.kroʊˈpɔːr.əs/
Phonetic Spelling: my·kroh·por·uhs
6. Xcalibur
Pronunciation link: <https://www.howtopronounce.com/xcalibur>
IPA: /ˈɛksˈkæl.ɪ.bər/
Phonetic Spelling: eks·kal·uh·bur
7. Spectrometry
Pronunciation link: <https://www.merriam-webster.com/dictionary/spectrometry>
IPA: /ˈspekˈtrɔː.mə.tri/
Phonetic Spelling: spek·traa·muh·tree
8. Mass-to-charge
Pronunciation link: <https://www.howtopronounce.com/mass-to-charge-ratio>
IPA: /ˌmæs təˈtʃɑːrdʒ/
Phonetic Spelling: mass tuh chaarj
9. Chromatogram
Pronunciation link: <https://www.merriam-webster.com/dictionary/chromatogram>
IPA: /ˌkroʊˈmæt.ə.ɡræm/
Phonetic Spelling: kroh·mat·uh·gram
10. Tandem
Pronunciation link: <https://www.merriam-webster.com/dictionary/tandem>
IPA: /ˈtæn.dəm/
Phonetic Spelling: tan·duhm

11. Fragmentation
Pronunciation link: <https://www.merriam-webster.com/dictionary/fragmentation>
IPA: /ˈfræɡ.mənˈteɪ.jən/
Phonetic Spelling: frag·muhn·tay·shuhn
12. Elucidation
Pronunciation link: <https://www.merriam-webster.com/dictionary/elucidation>
IPA: /ɪˈluː.səˈdeɪ.jən/
Phonetic Spelling: ih·loo·suh·day·shuhn
13. Precursor
Pronunciation link: <https://www.merriam-webster.com/dictionary/precursor>
IPA: /priˈkɜː.sə/
Phonetic Spelling: pree·kur·sur
14. Hexose
Pronunciation link: <https://www.merriam-webster.com/medical/hexose>
IPA: /ˈhek.sʊs/
Phonetic Spelling: hek·sohs
15. Rhamnose
Pronunciation link: <https://www.merriam-webster.com/medical/rhamnose>
IPA: /ˈræm.noʊs/
Phonetic Spelling: ram·nohs
16. Neobyakangelicol
Pronunciation link: <https://www.howtopronounce.com/neobyakangelicol>
IPA: /ˌniː.ʊʊ.bjɑː.kænˈdʒel.ɪ.koːl/
Phonetic Spelling: nee·oh·byaa·kan·jel·ih·kaal
17. Quadrupole
Pronunciation link: <https://www.merriam-webster.com/dictionary/quadrupole>
IPA: /ˈkwɑː.drəˌpɒl/
Phonetic Spelling: kwaa·druh·pohl
18. Collision
Pronunciation link: <https://www.merriam-webster.com/dictionary/collision>
IPA: /kəˈlɪʒ.ən/
Phonetic Spelling: kuh·lizz·uhn
19. Multistage
Pronunciation link: <https://www.merriam-webster.com/dictionary/multistage>
IPA: /ˌmʌl.tiˈsteɪdʒ/
Phonetic Spelling: mul·tee·stayj
20. Fragment
Pronunciation link: <https://www.merriam-webster.com/dictionary/fragment>
IPA: /ˈfræɡ.mənt/
Phonetic Spelling: frag·muhnt