Submission ID #: 61031
Scriptwriter Name: Bridget Colvin
Project Page Link: https://www.jove.com/account/file-uploader?src=18614503

Title: Simultaneous Quantification of Selected Kynurenines Analyzed by Liquid Chromatography-Mass Spectrometry in Medium Collected from Cancer Cell Cultures

Authors and Affiliations: Ilona Sadok[1], Kamila Rachwał[1], and Magdalena Staniszewska[1]

[1]Laboratory of Separation and Spectroscopic Method Applications, Centre for Interdisciplinary Research, The John Paul II Catholic University of Lublin

Corresponding Author:
Magdalena Staniszewska
staniszkul@kul.lublin.pl

Co-authors:
ilonasadok@kul.lublin.pl
kamila.rachwal@up.lublin.pl

Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

Videographer: All screen captures provided, do not film

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length

Number of shots: **50**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Magdalena Staniszewska**: Kynurenines are associated with immune response regulation in several diseases, including cancer. Reliable and validated methods for identifying multiple kynurenines aid in the development of more effective therapies **[1]**.

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

REQUIRED:

1.2. **Ilona Sadok**: Our protocol uses liquid chromatography coupled with mass spectrometry to determine the different tryptophan metabolites generated by the kynurenine pathway in medium collected from cancer cell cultures **[1]**.

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

OPTIONAL:

1.3. **Ilona Sadok**: Unexperienced users may have some difficulty during the sample preparation or data acquisition and interpretation steps. By following our protocol and recommendations, one can avoid mistakes and obtain reliable data **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

Protocol

2. 3-Nitrotyrosine (3NT), Kynurenine (Kyn), 3-Hydroxykynurenine (3HKyn), 3-Hydroxyanthranilic Acid (3HAA), Xanthurenic Acid (XA)and Charcoal Treated Culture Medium Preparation

2.1. To prepare stock solutions of the reagents, weigh out 0.3 milligrams of each reagent in a vial to the highest accuracy **[1]** and dissolve each reagent in 300 microliters of the appropriate solvent to obtain 1 gram/liter-stock solutions of each reagent **[2-TXT]**.

2.1.1. WIDE: Talent adding reagent to vial on balance, with reagent container visible in frame

2.1.2. Talent adding solvent to vial, with solvent container visible in frame **TEXT: See text for suggested solvent details**

2.2. To prepare charcoal-treated culture medium, weigh out 280 milligrams of activated charcoal in a conical tube **[1]** and add 5 milliliters of the liquid medium prepared for culturing the cells of interest **[2]**.

2.2.1. Talent adding charcoal to tube on balance

2.2.2. Talent adding medium to charcoal

2.3. Shake the tube with the medium and charcoal on a seesaw rocker for 2 hours at room temperature and 50 oscillations/minutes **[1]**.

2.3.1. Tube shaking on shaker

2.4. At the end of the incubation, sediment the charcoal by centrifugation **[1-TXT]** and carefully collect the supernatant without disturbing the charcoal **[2]**.

2.4.1. Talent adding tube(s) to centrifuge **TEXT: 15 min, 6000 x g, RT**
2.4.2. Shot of sedimented charcoal, then supernatant being collected

2.5. Then filter the medium using a 0.45-micrometer syringe filter **[1]**.

2.5.1. Talent filtering medium

3. Calibration Solution Preparation

3.1. To prepare the calibration solution, spike the charcoal-treated culture medium with 0.75 microliters of 0.1 gram/liter 3NT (three-N-T) solution **[1]** and add 3H-kynurenine, 3HAA (three-H-A-A), kynurenine, xanthurenic acid to at least at six different concentrations to cover all of the calibration ranges to a final volume of 150 microliters per sample **[2]**.

3.1.1. WIDE: Talent adding 3NT to tube(s), with 3NT container visible in frame
3.1.2. Talent adding standard(s) to tube(s), with standard containers visible in frame

3.2. After vortexing, add 150 microliters of minus twenty-degree Celsius cold methanol supplemented with 1% formic acid to each tube for sample deproteinization **[1]** and tightly cap and vortex the tubes again **[2]**.

3.2.1. Talent adding 1% formic acid in methanol to tube(s), with 1% formic acid in methanol container~~s~~ visible in frame
3.2.2. Tube(s) being vortexed

3.3. After a 40-minute incubation at minus 20 degrees Celsius **[1]**, centrifuge the samples **[2-TXT]** and use an automatic pipette to transfer 270 microliters of each supernatant into individual, flat-bottom glass vials **[3]**.

3.3.1. Talent removing tube(s) from -20 °C
3.3.2. Talent placing tube(s) into centrifuge **TEXT: 15 min, 14,000 x g, 4 °C**
3.3.3. Talent adding supernatant to vial(s)

3.4. Place the vials into an evaporator **[1]** and use the appropriate program for water-methanol fractions to gently evaporate the volatile components for about 30 minutes **[2-TXT]**.

3.4.1. Talent placing vial(s) into evaporator
3.4.2. Talent setting program **TEXT: Do not dry >40 °C and avoid over drying**

3.5. When the tubes are dry, reconstitute each sample in 60 microliters of 0.1% formic acid in water **[1]** and vortex the samples **[2]** before transferring each solution into individual 1.5-milliliter tubes for centrifugation **[3-TXT]**.

3.5.1. Talent adding acid to tube(s), with acid container visible in frame *Videographer: Important step*
3.5.2. Sample(s) being vortexed *Videographer: Important step*
3.5.3. Talent add solution to tube and centrifuge *Videographer: Important step* **TEXT: 15 min, 14,000 x g, 4 °C**

3.6. Without disturbing the sediment, transfer the supernatants into chromatographic vials with conical glass inserts **[1]** and place the vials into an LC-MS (L-C-M-S) autosampler **[2-TXT]**.

3.6.1. Shot of vial with solution carefully handled, then supernatant being transferred into a vial with conical glass inert and avoiding air bubble generation *Videographer: Important/difficult step*

3.6.2. Talent placing vial(s) into autosampler *Videographer: Important/difficult step* **TEXT: LC-MS: liquid-chromatography-mass spectrometry**

4. Liquid Chromatography-Mass Spectrometry (LC-MS) System Setup

4.1. Before running the samples, purge the LC system with the mobile phase to remove bubbles and to prime all of the solvent channels **[1]**

4.1.1. WIDE: Talent purging LC system

4.2. Next, flush the guard and analytical column with 100% acetonitrile for about 30 minutes **[1]** before flushing the system with 100% solvent A until a stable pressure is observed in the column **[2].**

4.2.1. Talent flushing with acetonitrile

4.2.2. Talent flushing with solvent A

4.3. Then set the appropriate LC parameters in the data acquisition software**[1-TXT]** and construct a worklist for running the samples on the LC system **[2]**.

4.3.1. Talent setting parameters, with monitor visible in frame **TEXT: See text for full parameter setup details**

4.3.2. SCREEN: screenshot_1

5. Calibration Curve Construction

5.1. To construct the calibration curve, in the data acquisition software **[1]**, add the calibration standards into the worklist and run the standards in triplicates **[2]**.

5.1.1. Talent opening data in software, with monitor visible in frame

5.1.2. SCREEN: screenshot_2 *Video Editor: please speed up*

5.2. Then integrate and measure the peak area corresponding to 3-hydroksykynurenine, kynurenine, 3-hydroxyanthranilic acid, 3-nitrotyrosine, and xanthurenic acid at the retention time of about 4.4-, 10-, 16-, 21-, and 30 minutes, respectively **[1]**.

5.2.1. SCREEN: screenshot_3 *Video Editor: can speed up*

6. Sample Collection

6.1. To collect supernatant samples from an in vitro cell culture, after 48 hours of standard cell culture of the cells of interest **[1]**, transfer 500 microliters of supernatant from each well into individual 1.5-milliliter tubes **[2]** and remove the cell debris by centrifugation **[3-TXT]**.

6.1.1. WIDE: Talent removing plate from incubator
6.1.2. Talent adding supernatant to tube(s)
6.1.3. Added shot: Talent placing tube(s) into centrifuge
TEXT: Collect cells remaining on plates by standard trypsinization

6.2. Then transfer the medium supernatants into new tubes for minus 80-degree Celsius storage until the analysis **[1]** and maintain the cell pellets at minus 20-degree Celsius until protein estimation analysis **[2]**.

6.2.1. Talent adding supernatant to tube(s)
6.2.2. Talent placing cell pellet tube(s) at -20 °C

7. LC-MS Analysis Sample Preparation

7.1. To prepare samples for LC-MS analysis, transfer 149.25 microliters of each thawed culture medium sample into a new 1.5-microliter tube **[1]** and add 0.75 microliters of 0.1 gram/liter 3NT solution to each tube **[2]**.

7.1.1. WIDE: Talent adding sample(s) to tube(s)
7.1.2. Talent adding 3NT to tube(s), with 3NT containers visible in frame

7.2. After calibrating the samples as demonstrated, add 150 microliters of minus 20-degree Celsius-methanol supplemented with 1% formic acid to each tube **[1]** and prepare the sample for LC-MS analysis as demonstrated **[2-TXT]**.

7.2.1. Talent adding 1% formic acid in methanol to tubes, with 1% formic acid in methanol container visible in frame
7.2.2. Talent placing tube(s) at -20 °C. **TEXT: Calibrate solutions as previously demonstrated**

7.3. When the worklist is complete, measure the peak area of each analyte **[1-TXT]** and use an individual linear calibration equation dedicated for each analyte to calculate concentrations of the analytes present within each experimental sample **[2]**.

7.3.1. Talent at computer, measuring peak area, with monitor visible in frame **TEXT: See text for peak area measurement details**
7.3.2. SCREEN: screenshot_4 *Video Editor: please speed up*

8. Protein Content Assessment and Data Normalization

8.1. To assess the protein content within the cell culture medium samples, resuspend each cell pellet in 100 microliters of PBS **[1]** and freeze the samples at minus 20 degrees Celsius for 1 hour **[2]** before thawing them on ice **[3]**.

8.1.1. WIDE: Talent adding PBS to tube(s), with PBS container visible in frame *Videographer: Important step*
8.1.2. Talent placing tube at -20 °C *Videographer: Important step*
8.1.3. Talent placing tube on ice *Videographer: Important step*

8.2. After freeze-thawing the samples three times, collect the cell lysates by centrifugation **[1]** and transfer the supernatants into new tubes without disturbing the pellets **[2]**.

8.2.1. Talent placing tube(s) into centrifuge
8.2.2. Talent adding supernatant(s) to tube

8.3. Dilute the samples 10 times with fresh PBS **[1]** and add the appropriate volumes of bovine serum albumin standard solution in duplicate to the appropriate wells of a 96-well plate **[2-TXT]**.

8.3.1. Talent adding PBS to sample tube(s), with PBS container visible in frame
8.3.2. Talent adding standard to well(s), with BSA container visible in frame **TEXT: *e.g.*, 0-, 2-, 4-, 5-, 7-, 10-, 15-, and 20-microliters**

8.4. Load 10-15 microliters of each cell lysate sample supernatant to the appropriate wells of the 96-well plate **[1]** and fill both the standard and sample wells to a final volume of 50 microliters of PBS per well **[1]**.

8.4.1. Talent adding sample(s) to well(s), with sample tubes visible in frame
8.4.2. Talent adding PBS to well(s), with PBS container visible in frame

8.5. Next, add 200 microliters of a Bradford reagent diluted 5 times with ultrapure water to each well **[1]** and incubate the plate for at least 5 minutes at room temperature **[2]**.

8.5.1. Talent adding reagent to well(s), with reagent container visible in frame
8.5.2. Talent setting timer, with plate on bench visible in frame

8.6. At the end of the incubation, load the plate onto a microplate reader **[1]** and measure the absorbance at 595 nanometers **[2]**.

8.6.1. Talent loading plate
8.6.2. Talent measuring absorbance, with monitor visible in frame

8.7. Then construct a calibration curve to allow calculation of the amount of protein in each sample **[1]** and divide the concentration of each kynurenine by the total protein content to normalize the kynurenine amount in each sample per 1 microgram of protein **[2]**.

8.7.1. SCREEN: Screenshot_5 *Video Editor: please speed up*
8.7.2. SCREEN: Screenshot_6 *Video Editor: please speed up*

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
3.5., 3.6., 8.1.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
3.6.

Results

9. Results: Representative LC-MS Analysis of Selected Kyn

9.1. Here single quadrupole mass spectrometry results obtained during the analysis of medium containing 4.5 grams/liter of D-glucose and 10% fetal bovine serum **[1]**. Note the small peak indicating the presence of kynurenine within the sample **[2]**.

9.1.1. LAB MEDIA: Figure 1A
9.1.2. LAB MEDIA: Figure 1A *Video Editor: please emphasize peak and/or add Kyn text and arrow*

9.2. Run a quality control sample before acquiring the actual sample data to confirm the appropriate retention times of the analytes **[1]**, as a shift in LC signals or mismatches can be observed **[2]**.

9.2.1. LAB MEDIA: Figure 2A
9.2.2. LAB MEDIA: Figure 2C *Video Editor: please add/emphasize red oval*

9.3. Co-eluting matrix components can generate ions with the mass to charge ratio selected for the target analytes **[1]**.

9.3.1. LAB MEDIA: Figure 3A

9.4. For example, in this analysis, the peak from the unknown compound appeared in the scan period during which the ion of mass to charge ratio 206 was monitored **[1]**. Due to incompatibility of the retention time, the signal did not correspond to the analyte **[2]**.

9.4.1. LAB MEDIA: Figure 3A*Video Editor: please emphasize compound X peak and/or add/emphasize compound X text and arrow*
9.4.2. LAB MEDIA: Figure 3A *Video Editor: please emphasize XA peak and/or add/emphasize XA text and arrow*

9.5. Although the tryptophan isotope shared the same ion as mass to charge ratio 206 **[1]**, chromatographic analysis revealed that the tryptophan signal **[2]** was well separated from the xanthurenic acid signal, indicating that the level of tryptophan present in the tested cancer cell medium did not impact xanthurenic acid quantification **[3]**.

9.5.1. LAB MEDIA: Figure 4
9.5.2. LAB MEDIA: Figure 4 *Video Editor: please emphasize Trp peak and/or add/emphasize Trp text and arrow*

9.5.3. LAB MEDIA: Figure 4 *Video Editor: please emphasize XA peak and/or add/emphasize XA text and arrow*

9.6. This analysis of the culture medium from two types of cancer cell lines demonstrates that the evaluated human epithelial breast cancer cells released different amounts of tryptophan metabolites **[1]**, while the tested human ovarian cancer cells produced significantly higher levels of kynurenine **[2]**.

9.6.1. LAB MEDIA: Figure 3

9.6.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize Kyn peak*

Conclusion

10. Conclusion Interview Statements

10.1. **Ilona Sadok**: Using an internal standard during the sample preparation helps with reliable data acquisition. Normalization of the data to the protein content corrects for cell number variabilities within individual wells **[1]**.

10.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (8.3., 9.11.)

10.2. **Magdalena Staniszewska**: Our protocol can be further expanded to determine additional analytes that may accumulate in the culture medium under different experimental conditions **[1]**.

10.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*