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TITLE:

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

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liquid-chromatography mass spectrometry, tryptophan metabolites, kynurenine, 3-hydroxykynurenine, xanthurenic acid, 3-hydroxyanthranilic acid, cancer cells, sample preparation

SUMMARY:

Described here is a protocol for the determination of four different tryptophan metabolites generated in the kynurenine pathway (kynurenine, 3-hydroxykynurenine, xanthurenic acid, 3-hydroxyanthranilic acid) in the medium collected from cancer cell cultures analyzed by liquid chromatography coupled with a single quadrupole mass spectrometry.

ABSTRACT:

The kynurenine pathway and the tryptophan catabolites called kynurenines have received increased attention for their involvement in immune regulation and cancer biology. An in vitro cell culture assay is often used to learn about the contribution of different tryptophan catabolites in a disease mechanism and for testing therapeutic strategies. Cell culture medium that is rich in secreted metabolites and signaling molecules reflects the status of tryptophan metabolism and other cellular events. New protocols for the reliable quantification of multiple kynurenines in the complex cell culture medium are desired to allow for a reliable and quick analysis of multiple samples. This can be accomplished with liquid chromatography coupled with mass spectrometry. This powerful technique is employed in many clinical and research laboratories for the quantification of metabolites and can be used for measuring kynurenines.

Presented here is the use of liquid chromatography coupled with single quadrupole mass spectrometry (LC-SQ) for the simultaneous determination of four kynurenines, i.e., kynurenine, 3-hydroxykynurenine, 3-hydroxykynurenine, and xanthurenic acid in the medium collected

from in vitro cultured cancer cells. SQ detector is simple to use and less expensive compared to other mass spectrometers. In the SQ-MS analysis, multiple ions from the sample are generated and separated according to their specific mass-to-charge ratio (*m/z*), followed by the detection using a Single Ion Monitoring (SIM) mode.

This paper draws the attention on the advantages of the reported method and indicates some weak points. It is focused on critical elements of LC-SQ analysis including sample preparation along with chromatography and mass spectrometry analysis. The quality control, method calibration conditions and matrix effect issues are also discussed. We described a simple application of 3-nitrotyrosine as one analog standard for all target analytes. As confirmed by experiments with human ovary and breast cancer cells, the proposed LC-SQ analysis generates reliable results and can be further applied to other in vitro cellular models.

INTRODUCTION:

Kynurenine pathway (KP) is the major route of tryptophan (Trp) catabolism in human cells. Indoleamine-2,3-dioxygenase (IDO-1) in extrahepatic cells is the first and limiting enzyme of KP and converts Trp into N-formylkynurenine[1]. Further steps within KP generate other secondary metabolites, namely kynurenines that exhibit various biological properties. Kynurenine (Kyn) is the first stable Trp catabolite showing toxic properties and regulating cellular events after binding to the aryl hydrocarbon receptor (AhR)[2]. Subsequently, Kyn is transformed into several molecules either spontaneously or in the enzyme-mediated processes, generating such metabolites like 3-hydroxykynurenine (3HKyn), anthranilic acid (AA), 3-hydroxyanthranilic acid (3-HAA), kynurenic acid (Kyna), and xanthurenic acid (XA). Another downstream metabolite, 2-amino-3-carboxymuconic acid-6-semialdehyde (ACMS), undergoes non-enzymatic cyclization to quinolinic acid (QA) or picolinic acid (PA)[1]. Finally, QA is further transformed into nicotinamide-adenine dinucleotide (NAD^+)[3], the KP end-point metabolite that is an important enzyme cofactor. Some kynurenines have neuroprotective properties such as Kyna and PA, while the others, i.e., 3HAA and 3HKyn, are toxic[4]. Xanthurenic acid (XA), which is formed from 3HKyn, presents antioxidant and vasorelaxation properties[5]. XA accumulates in aging lenses and leads to apoptosis of epithelial cells[6]. KP, described in the middle of the 20th century, gained more attention when its involvement in various disorders was demonstrated. Increased activity of this metabolic route and accumulation of some kynurenines modulate the immune response and are associated with different pathological conditions such as depression, schizophrenia, encephalopathy, HIV, dementia, amyotrophic lateral sclerosis, malaria, Alzheimer's, Huntington's disease, and cancer[4,7]. Some changes in Trp metabolism are observed in tumor microenvironments and cancer cells[2,8]. Moreover, kynurenines are considered as promising disease markers[9]. In cancer research, in vitro cell culture models are well established and widely used for preclinical evaluation of responses to anticancer drugs[10]. Trp metabolites are secreted by cells into the culture medium and can be measured to assess the status of the kynurenine pathway. Therefore, there is a need to develop appropriate methods for the simultaneous detection of as many KP metabolites as possible in a variety of biological specimens with an easy, flexible, and reliable protocol.

In this paper, we describe a protocol for the simultaneous determination of four kynurenine pathway metabolites: Kyn, 3HKyn, 3HAA, and XA, determined by LC-QS in a post-culture medium collected from cancer cells. In a modern analytical approach, liquid chromatography[13-16] is preferred for the simultaneous detection and quantification of the individual tryptophan catabolites, in contrast to biochemical nonspecific assays utilizing Ehrlich reagent[11,12]. At present, there are many methods available for kynurenines determination in human specimens, mainly based on liquid chromatography with ultraviolet or fluorescence detectors[13,17-19]. Liquid chromatography coupled with a mass spectrometry detector (LC-MS) seems more suitable for this type of analysis, due to their higher sensitivity (lower limits of detection), selectivity and repeatability.

Trp metabolites have already been determined in human serum, plasma and urine[13,20-23], however, the methods for other biological specimens, like cell culture medium are also desired. Previously, LC-MS was used for Trp-derived compounds in a medium collected after culturing of human glioma cells, monocytes, dendritic cells or astrocytes treated with interferon gamma (IFN-γ)[24-26]. Currently, there is a need for new validated protocols that can allow an assessment of several metabolites in different culture media, cells, and treatments used in cancer models.

The purpose of the presented method is to quantify (within one analytical run) four major kynurenines that can indicate abnormalities in KP. Presented here are critical steps of our recently published protocol for quantitative LC-SQ analysis of selected meaningful kynurenines using one internal standard (3-nitrotyrosine, 3NT) in the medium collected from in vitro cultured human cancer cells[27]. To our best knowledge, it is the first LC-SQ protocol for simultaneous quantification of 3HKyn, 3HAA, Kyn and XA in a culture medium obtained from the in vitro grown cells. Upon some modifications, the method might be further applied to study the changes in Trp metabolism in a broader range of cell culture models.

PROTOCOL:

1. Preparation of standard 3NT, Kyn, 3HKyn, 3HAA, XA stock solutions

1.1. Weigh the reagents in a vial with the highest accuracy (0.3 mg each). For better accuracy, scale up the reagents, adjusting the volume of the solvent according to step 1.2.

1.2. Dissolve the reagents in 300 µL of the solvent to obtain a stock solution of 1 g/L. Dissolve 3NT in 1% (v/v) formic acid (FA) in water; Kyn, 3HAA, and XA in dimethyl sulfoxide (DMSO); 3HKyn in water acidified to pH 2.5 with hydrochloric acid (HCl).

CAUTION: 3HAA irritates eyes, nose, throat and lungs. It is harmful when inhaled, in contact with skin, and if swallowed. Wear appropriate protection i.e., gloves and mask.

1.3 Tightly close the vial and place it in an ultrasonic bath for 1 min to accelerate dissolution.

NOTE: Store the stock solutions at -20 °C and minimize freezing/thawing (3 cycles max) due to the instability of the stock solutions.

2. Preparation of charcoal treated culture medium

2.1. In a tube, weigh 280 mg of activated charcoal and add 5 mL of the liquid medium prepared for culturing the cells of interest.

2.2. Shake the tube with the medium and charcoal on a see saw rocker for 2 h, at room temperature (set speed to 50 oscillations/min). Next, centrifuge, the tubes at 6000 x *g* for 15 min.

2.3. Remove the tube from the centrifuge and carefully collect the supernatant without disturbing the sediment. Repeat centrifugation if necessary, to remove all charcoal residues.

2.4. Filter the supernatant using a 0.45 µm syringe filter.

2.5. Ensure that the charcoal pretreated culture medium is deprived of kynurenines traces by running a pilot sample on LC-MS as described in step 6.4.2. Otherwise, repeat steps 2.1-2.4.

NOTE: If the complete culture medium initially does not contain kynurenines, purification step using charcoal might be omitted. In this case, prepare calibration standards and quality control samples using the complete medium usually prepared for cell culturing.

2.6. Store the prepared medium at 4 °C until analysis.

3. Making the calibration solutions and calibration curves

3.1. Spike the charcoal pretreated culture medium with 0.75 µL of 0.1 g/L 3NT solution and add four standards (3HKyn, 3HAA, Kyn, XA) at least at six different concentrations to cover calibration ranges. Keep the final volume of each sample at 150 µL. Vortex well. Use 1.5 mL centrifuge tubes for sample preparation.

NOTE: Suggested calibration points for 3HKyn: 0.018, 0.045, 0.22, 1.12, 2.23, 4.46 µmol/L; for Kyn: 0.0096, 0.048, 0.24, 1.20, 2.40, 3.84 µmol/L; for 3HAA: 0.033, 0.16, 0.65, 3.27, 6.53, 13.06 µmol/L; for XA: 0.019, 0.13, 0.49, 1.22, 3.65, 4.87 µmol/L.

3.2. Add 150 µL of cold methanol (kept at -20 °C) containing 1% (v/v) formic acid into each tube for sample deproteinization. Tightly close the tubes and vortex well.

3.3. Incubate the samples at -20 °C for 40 min.

3.4. Centrifuge the samples at 14,000 x *g* for 15 min at 4 °C. Remove the tubes from the centrifuge. Collect supernatants into the new tubes. Do not disturb the sediment.

3.5. Transfer 270 µL of the supernatant into the glass vial using an automatic pipette. Put the vials into the evaporator and gently evaporate until dry. Use the appropriate program for water/methanol fractions to evaporate volatile components. Do not apply temperature higher than 40 °C to avoid over-drying.

NOTE: Flat bottom glass vials, i.e., chromatographic vials facilitate faster evaporation and higher recoveries in comparison to plastic conical tubes.

3.6. After 30 min, check the evaporation status. If necessary, continue evaporation (e.g., add extra 10 min). Avoid over-drying.

3.7. Remove the vials from the evaporator. Reconstitute each sample in 60 µL of 0.1% (v/v) formic acid in water. Add the solvent into the vial containing the residual material. Tightly close the vials and vortex-well.

3.8. Transfer the sample into a 1.5 mL tube and spin at 14,000 x *g* for 15 min at 4 °C to separate the precipitated protein.

3.9. Without disturbing the protein pellet, transfer the supernatants into chromatographic vials with conical glass inserts with an automatic pipette. Tightly close the vials.

3.10. Check for the presence of air bubbles in the insert vial and remove them if necessary (e.g., by vortexing).

3.11. Transfer the chromatographic vials containing samples into LC autosampler. Record the position of samples placed in an autosampler tray.

4. Preparation of quality control (QC) samples

4.1. Spike the charcoal-pretreated culture medium (prepared in a 1.5 ml centrifugal tube) with 0.75 µL of 0.1 g/L 3NT solution and standards of four kynurenines (3HKyn, 3HAA, Kyn, XA) at one concentration selected from the linear range of the calibration curves. Keep the final volume of the sample at 150 µL.

NOTE: If applicable, prepare several QC samples at different concentrations falling under the linear range of the calibration curve.

4.2. Follow the protocol described in section 3 (steps 3.2-3.11).

5. Setting up the LC-MS system

5.1. Prepare the mobile phase solvents: Solvent A: 20 mmol/L ammonium formate in ultrapure water (pH 4.3 adjusted with formic acid); Solvent B: 100% acetonitrile.

NOTE: Use borosilicate glass bottles only. Rinse bottles with ultrapure water before refilling it. Do not use bottles cleaned with detergents.

CAUTION: Acetonitrile causes severe health effects or death. It is easily ignited by heat. Acetonitrile liquid and vapor can irritate eyes, nose, throat and lungs.

5.1.1. Prepare 5 mol/L stock solution of ammonium formate by dissolving a crystalline reagent (15.77 g) in 50 mL of ultrapure water in a glass bottle. Stir until all residuals dissolve. Filter through the 0.45 µm membrane to remove any residual debris (e.g., by using a nylon syringe filter). Store the stock solution at 4 °C.

5.1.2. Prepare Solvent A by adding 4 mL of 5 mol/L ammonium formate in water to 980 mL of ultrapure water in an amber glass bottle and stir well. Immerse a stirring bar and put the bottle with the solvent on a magnetic stirrer. Immerse a pH electrode into the solution and control the pH under stirring. Add formic acid stock solution (98%-100%) dropwise using an automatic pipette with 0.2 mL tip to obtain pH 4.3, adjust the volume up to 1 mL with ultrapure water and stir well.

NOTE: Freshly prepare the solvents at least once a week to prevent any microbial growth.

CAUTION: Formic acid (FA) is toxic when inhaled, causes skin burns and eye damage. Work under the fume hood; wear protective gloves and coat.

5.1.3. Filter all solutions through the 0.22 µm membrane (e.g., nylon syringe filter) to remove any residual debris. Optionally, use the solvent inlet filters dedicated for LC solvent reservoirs to protect the system from incoming particles.

5.2. Start LC-MS control and data acquisition software.

5.3. Purge the LC system with the mobile phase to remove bubbles, and to prime all the solvent channels.

5.4. Ensemble a guard column to protect the analytical column from clogging.

5.5. Flush the guard and analytical column (C18, 2.1 x 100 mm, 1.8 µm) with 100% acetonitrile for about 30 min, and then with 100% solvent A until a stable pressure in the column is observed. Control the system performance using the control software.

5.6. Set the appropriate LC parameters in the data acquisition software.

5.6.1. Set up the gradient program: 0-8 min solvent B 0%; 8-17 min solvent B 0-2%; 17-20 min solvent B 2%; 20-32 min solvent B 10-30%; 32-45 min solvent B 0%.

5.6.2. Set the mobile phase flow rate at 0.15 mL/min, total analysis run time for 45 min, column temperature at +40 °C and injection volume at 10 µL.

5.7. Set the acquisition parameters of the mass spectrometry detector.

5.7.1. Apply ionization parameters: single ion monitoring (SIM), positive ionization, nebulizer pressure of 50 psi, +350 °C drying gas temperature, drying gas flow of 12 L/min, and 5500 V capillary voltage.

5.7.2. Select the analyte monitoring ions: for 3HKyn *m/z* 225.0 (scan period: 2-6 min, fragmentor voltage: 100 V); for Kyn *m/z* 209.0 (scan period: 2-12 min, fragmentor voltage: 80 V); for 3HAA *m/z* 154.0 (scan period: 12-18 min, fragmentor voltage: 80 V); for 3NT *m/z* 227.0 (scan period: 18-23 min, fragmentor voltage: 100 V); for XA *m/z* 206.0 (scan period: 23-45 min, fragmentor voltage: 100 V).

5.8. Construct a worklist and run samples on the LC system.

5.9. At first, before the analysis of the experimental samples, run a blank sample (Solvent A) at least in triplicates, followed by one QC sample.

5.10. Open the data analysis software and load the results obtained for the QC sample.

5.11. Check the position of analyte peaks on the chromatogram. When signals shift beyond the expected position adjust the time gates for appropriate analyte signals collection. See the software manual for instruction on signal integration.

6. Constructing the calibration curve

6.1. In the data acquisition software, add calibration standards into the worklist and run the standards in triplicates.

6.2. Integrate and measure the peak area corresponding to 3HKyn, Kyn, 3HAA, 3NT, and XA at the retention time of about 4.4 min, 10 min, 16 min, 21 min, and 30 min, respectively.

6.3. Construct individual calibration curves for each metabolite using a spreadsheet program.

6.3.1. To create the calibration chart, plot the value for a ratio of analyte peak area over the 3NT peak area versus the concentration of the analyte.

6.3.2. Analyze the blank sample (charcoal-pretreated culture medium spiked only with 3NT) to ensure there is no trace of the studied analytes in the medium. Otherwise, subtract the obtained value from each calibration point.

6.3.3. Check the linearity of each calibration curves.

6.3.3.1. Individually, for each analyte, create a slope-intercept linear equation (y = ax +b), where *y* corresponds to the ratio of the analyte peak area versus 3NT peak area, *a* is the slope, *x* is the analyte concentration (µmol/L), and *b* refers to the curve intercept. Plotting the graph 'y' versus 'x' will generate the calibration curve.

6.3.3.2. Add a linear trendline to the chart, display the calibration curve equation and regression coefficient on the chart. Ensure that the regression coefficient is > 0.990. In case of mismatched calibration standards, prepare and/or analyze these once again. Optionally, change the concentration range of the calibration curve.

6.3.4. Analyze the QC samples to check the system performance before analyzing the experimental samples. In case of incompatibilities (± 20% deviation from the reference value), prepare the new calibration curves.

7. In vitro cell culture and sample collection for analysis

7.1. Plate the studied cancer cells (MDA-MB-231 or SK-OV-3) in DMEM (Dulbecco's Modified Eagle's Medium) containing 4.5 g/L of D-glucose, 10% (v/v) fetal bovine serum (FBS), 2 mmol/L L-glutamine and 1 U penicillin/streptomycin and culture at 37 °C in a humidified atmosphere of 5% CO_2 to expand them for the experiment. Passage the cells at 80% of confluency.

7.2. Detach cells from the culture dish using trypsin, seed for the experiment on 12-well plates at a density of 0.3 × 10^6 cells per well and place in an incubator overnight.

7.3. The next day, aspirate the culture medium and add fresh DMEM with or without the addition of the studied compounds, depending on the experiment design.

7.4. After 48 h, collect the medium from above the cells (about 500 µL) into a 1.5 mL tube. Centrifuge at 14, 000 x *g* for 5 min to remove any cell debris. Collect the supernatant and store at -80 °C until the analysis.

7.5. Save the cellular pellet from step 7.4 for protein estimation. Freeze the samples at -20 °C.

NOTE: The results obtained for a medium from different wells should be normalized to total protein content to account for the variability in cell number in individual wells. Protein concentration can be assessed as described in step 9.

8. Prepare samples for LC-MS analysis

8.1. Thaw the frozen sample at room temperature and mix well. Transfer 149.25 µL of the culture medium into a centrifugation tube (1.5 mL).

8.2. Add 0.75 µL of 0.1 g/L of 3NT solution (internal standard). Close the tubes and vortex well.

8.3. Add 150 µL of chilled at -20 °C methanol containing 1% (v/v) FA for sample deproteinization. Close the tubes and vortex well.

8.4. Continue with sample preparation as described in section 3 (steps 3.3-3.11).

NOTE: Some cancer cells like SK-OV-3 secrete large amounts of Kyn into a culture medium. To fit the data into a linear range of the calibration curve, approximately 100-fold dilution of the sample is necessary. In this case, dilute a portion of the sample with 0.1% (v/v) FA in water and analyze in addition to the undiluted sample. The reference samples of standards for the calibration curve should be prepared in 100 times diluted complete culture medium. Alternatively, add more points in the calibration curve (if proper solubility and linearity are achieved) to adjust it to the concentration level of Kyn in the experimental sample.

8.5. Analyze the samples by LC-MS as described in section 5. Construct a worklist and run each sample in triplicate.

8.6. After the worklist is completed, measure the peak area of analyte.

8.7. Generate a spreadsheet using the available software and the obtain numerical data.

8.8. In one spreadsheet column calculate the ratio of the analyte peak area over the 3NT peak area.

8.9. Use an individual linear calibration equation dedicated for each analyte (from step 6.4.3.) to calculate concentrations of analytes present in the experimental samples.

9. Assessing protein content and data normalization

9.1. Resuspend the cellular pellet from step 7.5 with 100 µL of phosphate-buffered saline (PBS) in 1.5 mL tube.

NOTE: Prepare PBS solution by dissolving 8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of sodium phosphate dibasic, potassium dihydrogen phosphate in 950 mL of ultrapure water. Stir well. Adjust the pH to 7.4 with hydrochloric acid (dropwise). Adjust volume up to 1 L with ultrapure water. Stir well until homogeneous.

CAUTION: Hydrochloric acid is highly corrosive and must be handled with suitable safety precautions. Contact with human skin can cause redness, pain, skin burns. Work under the fume hood; wear protective gloves and coat.

9.2. Freeze the samples at – 20 °C and then defrost on ice. Repeat this step 3x.

9.3. Centrifuge the samples at 14, 000 x *g* for 15 min at 4 °C. Collect the supernatants into the new tubes. Do not disturb the pellet.

9.4. Dilute the supernatants 10x with PBS.

9.5. Construct a calibration curve from 0 to 2.0 µg of the protein per well range.

9.5.1. Prepare bovine serum albumin (BSA) standard solution for the calibration. Dissolve 1.23 µg of BSA in 1 mL of ultrapure water and vortex well.

9.5.2. On a 96-well plate, load different amounts of BSA standard solution (1.23 µg/mL): 0 µL, 2 µL, 4 µL, 5 µL, 7 µL, 10 µL, 15 µL, 20 µL.

9.5.3. Fill the well with PBS to the final volume of 50 µL.

9.6. Load 10 - 15 µL cell lysate from step 9.2 on the same 96-well plate. Fill the wells with PBS to reach to the final volume of 50 µL.

NOTE: If needed, adjust the volume of the cell lysate aliquot in each well to fit in the linear range of the calibration curve. Keep the final volume to 50 µL of the sample per well.

9.7. Add 200 µL of a Bradford reagent (diluted 5-times with ultrapure water) to each well.

9.8. Incubate the 96-well pate for at least 5 min at room temperature. Insert the plate into a microplate reader. Measure absorbance at λ = 595 nm.

9.9. Construct a calibration curve using a spreadsheet program. Plot the BSA amount (µg) versus absorbance. Add a linear trendline, display the calibration curve equation and regression coefficient on the chart.

9.10. Use the linear equation (y = ax +b, where *y* corresponds to the absorbance at λ = 595 nm, *a* is the slope, *x* is a protein content (µg), and *b* refers to the curve intercept) to calculate protein amount in the sample.

NOTE: Consider a sample dilution factor in calculations.

9.11. Use the estimated protein content to normalize kynurenine amount in the sample per 1 µg of protein. To do this, divide the concentration of kynurenines determined in step 8.9 with the total protein content from step 9.10.

REPRESENTATIVE RESULTS:

LC-MS presents indisputable advantages in the quantification of biologically active molecules, even though some components of complex specimens cause so-called matrix effects and compromise ionization of analytes. It leads to ion suppression or ion enhancement, strongly

decreasing accuracy and affecting a limit of detection/quantification of LC-MS, which is considered as a ''weak" point of the method. In our protocol, the ions are generated by electrospray ionization (ESI) in the positive mode, which was also employed in other studies on Trp metabolites determination[13]. ESI, however, is more prone to matrix effects than atmospheric pressure chemical ionization (APCI)[28]. Thus, for accurate quantification of Trp metabolites in a cell culture medium, we used an internal standard and matrix-matched calibration to compensate the matrix-dependent effects on the analyte signal and to correct the loss of the target compounds during a sample preparation step. We applied the same internal standard (3NT) for all the studied analytes (3HKyn, Kyn, 3HAA, XA). 3NT was not found in the original, fresh medium used for cell culturing and shows a similar behavior like target compounds under the applied analytical conditions. That makes 3NT the appropriate internal standard[27]. In addition, to simplify the protocol, in order to make it more accessible to a wide group of users, we propose only one step of sample preparation involving protein removal by treatment with methanol containing 1% (v/v) FA.

Within the presented protocol, it is recommended to prepare the calibration curves using the complete medium used for cell culture. However, the culture medium might contain endogenous Kyn that disturbs proper estimation of the analyte peak area, especially in the calibration solutions at low concentrations. **Figure 1A** illustrates LC-SQ results obtained during the analysis of DMEM containing 4.5 g/L D-glucose (DMEM-HG) and 10% (v/v) FBS used by our group for a cancer cell culture. We have found that the fresh complete culture medium initially contains some Kyn that can be removed by purification with activated charcoal (**Figure 1B**). While analyzing the experimental samples, the complete DMEM-HG culture medium was also analyzed as a control sample, and the Kyn peak area was subtracted from the peak area recorded for Kyn in each tested sample.

[Place **Figure 1** here]

In the following step, a retention time for each target analyte was established (see a chromatogram in **Figure 2A)**. The good practice in LC-MS analysis is to run a QC sample before the actual samples to confirm appropriate retention times of the analytes. Occasionally, a shift in LC signals can be observed, and mismatches tend to happen. **Figure 2B** shows a slight change in the retention times of the analytes, which, however, does not disturb correct measurements. On the other hand, **Figure 2C** presents a significant change in a position of the target peaks. As seen, 3NT signal was significantly shifted towards a shorter retention time and came out of the set time gate. In this case, an appropriate quantitative analysis was impossible because the internal standard signal cannot be measured correctly. This might be due to several factors, i.e. insufficient column equilibration, incorrect mixing of the solvents in the pump, use of inappropriate sample diluent or contamination of the column stationary phase. **Figure 2D,** however, represents a situation, when the registered peaks suffer from severely low intensities. This can be a result of an injection failure, a leak in the system or MS damage. Furthermore, the co-eluting matrix components can generate ions with *m/z* selected for the target analytes, like in **Figure 3A,** where the result from the analysis of MDA-MB-231 cells culture medium are shown. At the retention time of ca 25 min, a strong signal derived from an unknown matrix

component (compound X) was observed. The peak appears in the scan period, when the ion of *m/z* 206 (selected for XA) was monitored. However, this signal does not correspond to the analyte due to incompatibility of the retention time. To avoid mistakes during peak integration, a comparison of the retention time with that one recorded for QC samples is also recommended.

[Place **Figure 2** and **Figure 3** here]

The culture medium used for cancer cells (DMEM) contains significant amounts of Trp. Its isotope (^{13}C-Trp) shares the same ion as XA (*m/z* 206) and might interfere with XA determination. However, under the applied chromatographic conditions, the signal from Trp is well separated from the XA signal. Therefore, we concluded that Trp present in DMEM does not affect XA quantification and accuracy of the method (**Figure 4**).

[Place **Figure 4** here]

The presented analytical method successfully passed the validation test in terms of linearity, precision (coefficients of variation ≤15%), accuracy (96-104%), recovery (96-119%), and matrix effects for all analytes, like it was described in detail in our previous paper [27].

Finally, we confirmed an application of the described analytical approach to a medium collected from the in vitro cultured human cancer cells. Our data confirmed that a level of kynurenines can significantly vary in a culture medium collected from different cell types (**Figure 3**). We analyzed the culture medium from 2 types of cancer lines i.e. MDA-MB-231 and SK-OV-3 cells derived from breast and ovarian cancer, respectively. The selected lines are often used as models to study molecular events and for anti-cancer drug testing. As we observed, the cells cultured in a control standard medium released different amounts of tryptophan metabolites, i.e., MDA-MB-231 cells released a quantifiable amount of Kyn and XA at low nmol/L concentration (**Figure 3A),** while SK-OV-3 cells showed significantly more Kyn, very low secretion of XA, and no secretion of other studied kynurenines as indicated by the intensity of the corresponding peaks (**Figure 3B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Representative LC-SQ results of complete DMEM-HG culture medium (A) before and (B) after pretreatment on activated charcoal. Samples of culture medium were spiked with the same amount of the internal standard (3NT).

Figure 2: Examples of correct and incorrect results. The correct chromatograms of QC sample analysis at (**A**) medium and (**B**) low concentration levels recorded in different days. Example of the incorrect chromatogram resulting from significant change in the retention times of the analytes (**C**) and unsatisfactory intensities of peaks (**D**).

Figure 3. Representative results of culture medium collected from different cancer cell lines. Culture medium from (**A**) MDA-MB-231 (human breast cancer) and (**B**) SK-OV-3 (human ovary

cancer) cells was analyzed using LC-SQ. The compound X indicates the signal of an unknown compound present in the culture medium that co-elutes with the analyte and ionizes to form an ion with *m/z* selected for the analyte.

Figure 4. Position of tryptophan (Trp) and xanthurenic acid (XA) signals on MS chromatogram. Standard solutions of Trp (49.0 µmol/L) and XA (48.8 µmol/L) were prepared in Solvent A (20 mmol/L ammonium formate in water, pH 4.3) under optimized LC-SQ conditions. The ions of *m/z* 205 (corresponding to $[Trp+H]^+$) and *m/z* 206 (corresponding to $[XA+H]^+$) were monitored for Trp and XA, respectively.

DISCUSSION:

This paper presents a detailed LC-SQ protocol for the simultaneous quantification of four major Trp metabolites (3HKyn, Kyn, 3HAA, XA) that are measured in a medium from in vitro cultured human cancer cells. Special attention is paid to the sample preparation, chromatographic/mass spectrometry procedure, and data interpretation, the most important points within the analysis.

In general, LC-MS analysis, due to high sensitivity, requires the highest standards of a protocol strictness and purity of used materials. It refers to an application of neatly cleaned and properly washed glassware, as well as high grade chemicals throughout the standard procedure. It is very important to use fresh water-based solvents of a mobile phase to avoid microbial contamination that in this sensitive approach might drastically affect the analysis. Before injecting into an LC system, the analyzed samples need to be carefully examined for the presence of any insoluble particles. It is important to note that the column should be sufficiently equilibrated before the sample analysis. Failure to comply with these rules can result in the contamination of the samples, the LC system, and the analytical column or in insufficient sample ionization in MS source, finally causing a shift of signals.

There are 2 key points within the protocol that need to be emphasized in order to get optimal results. The most critical part in the presented protocol is the sample preparation step. Using an inadequate procedure causes a loss of target compounds and consequently inaccurate quantification. In our approach, during the method development, sample preparation step was carefully optimized along with the selection of the solvent for protein removal. As we determined, sample treatment with methanol containing 1% (v/v) formic acid yielded the best recoveries for all studied kynurenines. The impact of a mobile phase composition on the extent of analyte ionization was also evaluated. We have checked mobile phases consisting of 0-20 mmol/L ammonium formate or ammonium acetate at different pH adjusted with formic or acetic acid. The mobile phase containing 20 mmol/L ammonium formate in water (pH 4.3, solvent A) and acetonitrile (solvent B) provided the best possible conditions for the simultaneous quantification of Kyn, 3HKyn, 3HAA and XA[27]. Another important step in LC-MS analysis is peak integration. Some kynurenines occur in a sample at very low concentration levels. In this case, the signals of co-eluting compounds might overlap with the target analyte signal or generate a peak at a similar retention time. An unexperienced user of this method may find some difficulties with a correct peak integration, as exemplified in **Figure 2C**. Thus,

checking retention time for the analytes and comparing with a QC sample is required and highly recommended.

Good analytical practice includes selecting an appropriate internal standard for a quantitative analysis. Herein, we propose a simple and cost effective approach using only one internal standard (3NT) as an analogue of four kynurenines. The results confirmed that 3NT presents similar chromatographic behavior like the target compounds and in result it allows achieving a good precision and accuracy of the method[27]. Under the applied LC conditions, 3NT peak is well separated from the other analytes' signals and easy to measure. We realize that isotopically labeled internal standards (ILIS) used in such analyses[14-15] will provide better compensation of matrix effects and better control of all the variables that can lead to false results. On the other hand, ILISs for all target analytes are not always easily available, let alone their high cost. Also, ILISs are dedicated rather for LC-MS/MS than LC-SQ with Single Ion Monitoring (SIM) mode that we employed in this paper.

Using 3NT as an internal standard might be considered here as a limitation of the method due to a possibility of 3-nitrotyrosine formation on proteins[29]. However, in case of a culture medium, we did not observe any free endogenous 3-nitrotyrosine. It let us recognize 3NT as a suitable internal standard. However, we recommend a prior assessment of an initial endogenous 3NT level, especially when studying other cell types than tested in this report.

In summary, when selecting an analog of any analyte, one needs to consider many features, e.g., chemical similarity and initial absence in a sample matrix. Furthermore, a stability of the analogue internal standard in a sample matrix, its recovery and ionization efficiency in presence of matrix components might differ from the target compounds. Thus, all these features should be considered and carefully investigated during method development.

The analytical system in the presented method comprising an MS detector allowed us to achieve low limits of detection (0.0033 – 0.0108 µmol/L)[27]. This way simultaneous measurements of 3HKyn, Kyn, 3HAA, XA within the concentration ranges of 0.018 - 4.46 µmol/L, 0.0096 - 3.84 µmol/L, 0.033 - 13.06 µmol/L, and 0.019 – 4.87 µmol/L, respectively, was achieved. The main limitation of any LC-SQ analysis is associated with a proper separation of sample components on an LC column. Poor separation contributes to lower selectivity compared to the detection with a tandem quadrupole mass spectrometry utilizing product ions and Multiple Reaction Monitoring (MRM) mode. In order to avoid interferences from other matrix components that share the same or similar ions with a target molecule, the LC separation conditions must be carefully optimized. As an example, ^{13}C Trp isotope (present in trace amounts) generates the same ion of *m/z* 206 as selected for XA monitoring. The misinterpretation was avoided here by optimizing the chromatographic conditions and satisfactory separation of Trp and XA eluted from the column (**Figure 4**).

In the future applications, some modifications to the protocol can be implemented. A potential user can modify a concentration of the internal standard (3NT) when the levels of studied kynurenines are expected to fall beyound the concentrations presented here. Importantly, 3NT

concentration should be the same both in the calibration standards and in the experimental samples. In case of an extremely high analyte level, such as Kyn observed in SK-OV-3 cells, we recommend working with a higher concentration of 3NT. If sample dilution is required, it shoud be done at the final step of the protocol, before sample injection onto the LC column.

In the literature, there are some protocols proposed for LC-MS/MS quantitative analysis of kynurenines in a culture medium from different cells. One protocol provides a simultaneous determination of 3 metabolites, i.e., Kyn, 3HKyn and 3HAA but it is less sensitive (limit of quantifications (LOQ) at higher levels), in contrast to our method[21]. Another report presented a method that allows quantification of 4 kynurenines, including Kyn, 3HKyn, 3HAA and XA with similar LOQs[25]. None of them, however, was optimized for the detection of kynurenines generated by in vitro cultured cancer cells. The components of a sample matrix influence the analyte ionization in MS source by decreasing or increasing the signal, which is a cause of unreliable results. To obtain more accurate data, we proposed to use an analogue internal standard (3NT) in combination with a matrix-matched calibration for better compensation of matrix-dependent effects.

Using the presented methodology, we could observe that Kyn was the most abundant Trp metabolite in a culture medium collected from the analyzed cancer cell types, while the other studied metabolites under control culture conditions were not detected (excluding XA present in trace but detectable amounts). However, when the cells were stimulated with a potent immune activator – bacterial lipopolysaccharide, a larger amount of XA in addition to Kyn was secreted. On the other hand, the detected 3HKyn and 3HAA that are formed upstream of XA within KP were still under LOQ[27]. This suggests that some KP metabolites present in small amounts in a culture medium might be difficult to measure using the proposed LC-SQ approach. Nevertheless, the presented protocol is useful to identify changes in KP by quantification of the accumulating key Trp metabolites. Employing this methodology in future studies to engage an in vitro cell culture system will deliver novel biomarkers of immune-related diseases and cancer. Our approach brings a validated and reliable assay with relevant sensitivity for cellular models that are challenging due to low concentrations of the downstream KP metabolites. The provided instructions will allow researchers from different fields to utilize this approach for quantification of major kynurenines related to cancer and other diseases. Our data (unpublished) show that it is useful for studying KP modulation in cells exposed to different glycation products, but it also should find an application in other research fields and diseases with immune component, i.e., in endometrium biology and reproduction[30].

The presented protocol can be further expanded to determine additional analytes that might accumulate in a culture medium during different experimental conditions. The appropriate reference standards of analytes should be employed for method validation in terms of accuracy, precision, linearity, recovery or selectivity before being used for quantitative analyses. Futhermore, depending on the research purpose, the protocol might be used for individual kynurenines (3HKyn, Kyn, 3HAA or XA). In this case, the ions corresponding to compounds that are not considered for analysis, might be removed from the MS settings. In this case, suitable changes in the LC gradient program will help with cutting on analysis time.

When studying KP, it is relevant to assess the activity of IDO enzyme. This might be estimated simply by measuring Trp consumption and Kyn release into a culture medium or by calculating a kynurenine-to-tryptophan concentration ratio ([Kyn]/[Trp])[31]. In our approach, we have not measured a Trp concentration, however, the protocol might be expanded by adding this target in the LC-SQ analysis. As a more biochemically appropriate approach, we recommend to express IDO enzymatic activity as an amount of Kyn produced by cells per milligram of protein per minute as presented elsewhere[32]. We have noticed an advantage in using this approach in our other studies on cancer cells (manuscript in preparation) and recommend this method when using in vitro cell culture model.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Li, F., Zhang, R., Li, S., Liu, J. IDO1: an import ant immunotherapy target in cancer treatment. *International Immunopharmacology.* **47**, 70-77 (2017).
2. Heng, B. et al. Understanding the role of the kynurenine pathway in human breast cancer immunobiology. *Oncotarget*. **7** (6), 6506–6520 (2015).
3. Badawy, A. A. B. Kynurenine pathway and human systems. *Experimental Gerontology*. **129**, doi: 10.1016/j.exger.2019.110770 (2020).
4. Chen, Y., Guillemin, G. J. Kynurenine pathway metabolites in humans: Disease and healthy states. *International Journal of Tryptophan Research*. **2** (1), 1–19 (2009).
5. Sathyasaikumar, K. V. et al. Xanthurenic Acid Formation from 3-Hydroxykynurenine in the Mammalian Brain: Neurochemical Characterization and Physiological Effects. *Neuroscience*. **367**, 85–97 (2017).
6. Malina, H., Richter, C., Frueh, B., Hess, O. M. Lens epithelial cell apoptosis and intracellular Ca2+ increasein the presence of xanthurenic acid. *BMC Ophthalmology*. **2**, 1–7 (2002).
7. Colín-González, A. L., Maldonado, P. D., Santamaría, A. 3-Hydroxykynurenine: an intriguing molecule exerting dual actions in the central nervous system. *Neurotoxicology*. **34**, 189–204 (2013).
8. Xue, P., Fu, J., Zhou, Y. The aryl hydrocarbon receptor and tumor immunity. *Frontiers in Immunology*. 9 (286), 00286 (2018).
9. Tan, V. X., Guillemin, G. J. Kynurenine pathway metabolites as biomarkers for amyotrophic lateral sclerosis. *Frontiers in Microbiology*. **13** (1013), 1–11 (2019).
10. Wilding, J. L., Bodmer, W. F. Cancer cell lines for drug discovery and development.

Cancer Research. 14 (40), 2377-2384 (2015).
11. Takikawa, O., Kuroiwa, T., Yamazaki, F., Kido, R. Mechanism of interferon-gamma action. Characterization of indoleamine 2,3-dioxygenase in cultured human cells induced by interferon-gamma and evaluation of the enzyme-mediated tryptophan degradation in its anticellular activity. *Journal of Biological Chemistry*. **263** (4), 2041-2048 (1988).
12. Park, A. et al. Indoleamine-2,3-dioxygenase in thyroid cancer cells suppresses natural killer cell function by inhibiting NKG2D and NKp46 expression via STAT signaling pathways. *Journal of Clinical Medicine.* **8** (6), 842-859 (2019).
13. Sadok, I., Gamian, A., Staniszewska, M. M. Chromatographic analysis of tryptophan metabolites. *Journal of Separation Science*. **40** (15), 3020–3045 (2017).
14. Fuertig, R., et al. LC-MS/MS-based quantification of kynurenine metabolites, tryptophan, monoamines and neopterin in plasma, cerebrospinal fluid and brain. *Bioanalysis*. **8** (18), 1903–1917 (2016).
15. Marcos, J. et al. Targeting tryptophan and tyrosine metabolism by liquid chromatography tandem mass spectrometry. *Journal of Chromatography A*. **1434**, 91–101 (2016).
16. Möller, M., Du Preez, J., Harvey, B. Development and validation of a single analytical method for the determination of tryptophan, and its kynurenine metabolites in rat plasma. *Journal of Chromatography B: Analytical Technologies in Biomedical Life Science*. **898**, 121–129 (2012).
17. Lesniak, W. G. et al. Concurrent quantification of tryptophan and its major metabolites. *Analytical Biochemistry*. **443** (2), 222–231 (2013).
18. Badawy, A. A. B., Morgan, C. J. Rapid isocratic liquid chromatographic separation and quantification of tryptophan and six kynurenine metabolites in biological samples with ultraviolet and fluorimetric detection. *International Journal of Tryptophan Research*. **3**, 175-86 (2010).
19. Vignau, J., Jacquemont, M.C., Lefort, A., Imbenotte, M., Lhermitte, M. Simultaneous determination of tryptophan and kynurenine in serum by HPLC with UV and fluorescence detection. *Biomedical Chromatography*. **18** (10), 872–874, doi: 10.1002/bmc.445 (2004).
20. Zhang, A., Rijal, K., Ng, S. K., Ravid, K., Chitalia, V. A mass spectrometric method for quantification of tryptophan-derived uremic solutes in human serum. *Journal of Biological Methods*. **4** (3), 75, e75 (2017).
21. Arnhard, K.et al. A validated liquid chromatography-high resolution-tandem mass spectrometry method for the simultaneous quantitation of tryptophan, kynurenine, kynurenic acid, and quinolinic acid in human plasma. *Electrophoresis*. **39** (9–10), 1171–1180 (2018).
22. Oh, J. S., Seo, H. S., Kim, K. H., Pyo, H., Chung, B. C., Lee, J. Urinary profiling of tryptophan and its related metabolites in patients with metabolic syndrome by liquid chromatography-electrospray ionization/mass spectrometry. *Analytical and Bioanalytical Chemistry*. **409** (23), 5501–5512 (2017).
23. Huang, Y. et al. A simple LC-MS/MS method for determination of kynurenine and tryptophan concentrations in human plasma from HIV-infected patients. *Bioanalysis*. **5** (11), 1397–1407 (2013).
24. Yamada, K., Miyazzaki, T., Shibata, T., Hara, N., Tsuchiya, M. Simultaneous measurement of tryptophan and related compounds by liquid chromatography/electrospray ionization

tandem mass spectrometry. *Journal of Chromatography B: Analytical Technologies in Biomedical and Life Sciences*. **867** (1), 57–61 (2008).
25. Zhu, W. et al. Quantitative profiling of tryptophan metabolites in serum, urine, and cell culture supernatants by liquid chromatography-tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*. **401**, 3249–3261 (2011).
26. Hashioka, S. et al. Metabolomics analysis implies noninvolvement of the kynurenine pathway neurotoxins in the interferon-gamma- induced neurotoxicity of adult human astrocytes. *Neuropsychiatry*. **7** (2) (2017).
27. Sadok, I., Rachwał, K., Staniszewska, M. Application of the optimized and validated LC-MS method for simultaneous quantification of tryptophan metabolites in culture medium from cancer cells. *Journal of Pharmaceutical and Biomedical Analysis*. **176**, 112805–112815 (2019).
28. Annesley, T. M. Ion suppression in mass spectrometry. *Clinical Chemistry*. **49**(7) 1041-1044 (2003).
29. Houée-Lévin, C. et al. Exploring oxidative modifications of tyrosine: An update on mechanisms of formation, advances in analysis and biological consequences. *Free Radical Research*. **49** (4), 347–373 (2015).
30. Wei, H. et al. Abnormal Expression of Indoleamine 2, 3-Dioxygenase in Human Recurrent Miscarriage. *Reproductive Sciences*. e1933719119833788 (2019).
31. Liu, P. et al. Expression of indoleamine 2,3-dioxygenase in nasopharyngeal carcinoma impairs the cytolytic function of peripheral blood lymphocytes. *BMC Cancer*. **9**, 416 (2009).
32. Mailankot, M. *et al.* Indoleamine 2,3-dioxygenase overexpression causes kynurenine-modification of proteins, fiber cell apoptosis and cataract formation in the mouse lens. *Laboratory Investigation: A Journal of Technical Methods and Pathology*. **89** (5), 498–512 (2009).

Figure 1

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Figure 2

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Figure 3

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Figure 4

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Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
3-hydroksy-DL-kynurenina	Sigma-Aldrich	H1771	
3-hydroxyanthranilic acid	Sigma-Aldrich	148776	97%
3-nitro-L-tyrosine	Sigma-Aldrich	N7389	
Acetonitrile	Supelco	1.00029	hypergrade for LC-MS LiChrosolv
Activated charcoal	Supelco	05105	powder
Analytical balance	Ohaus		
Analytical column	Agilent Technologies	959764-902	Zorbax Eclipse Plus C18 rapid resolution HT (2.1 x 100 mm, 1.8 µm)
Ammonium formate	Supelco	7022	eluent additive for LC-MS, LiChropur, ≥99.0%
Bovine Serum Albumin	Sigma	1001887398	
Caps for chromatographic vials	Agilent Technologies	5185-5820	blue screw caps,PTFE/red sil sepa
Cell culture dish	Nest	704004	polystyrene, non-pyrogenic, sterile
Cell culture plate	Biologix	07-6012	12-well, non-pyrogenic, non-cytoxic, sterille
Cell incubator	Thermo Fisher Scientific		HERAcell 150i Cu
Centrifuge	Eppendorf		model 5415R
Centrifuge	Eppendorf		model 5428
Centrifuge tubes	Bionovo	B-2278	Eppendorf type, 1.5 mL
Centrifuge tubes	Bionovo	B-3693	Falcon type, 50 mL, PP
Chromatographic data acqusition and analysis software	Agilent Technologies		LC/MSD ChemStation (B.04.03-S92)
Chromatographic insert vials	Agilent Technologies	9301-1387	100 µL
Chromatographic vials	Agilent Technologies	5182-0714	2 mL, clear glass
Dimethyl sulfoxide	Supelco	1.02950	Uvasol
Dubelcco's Modified Eagle's Medium (DMEM)	PAN Biotech	P04-41450	
Dual meter pH/conductivity	Mettler Toledo		SevenMulti
Evaporator	Genevac		model EZ-2.3 Elite
Fetal bovine serum	Sigma-Aldrich	F9665	
Formic acid	Supelco	5.33002	for LC-MS LiChropur
Glass bottle for reagents storage	Bionovo	S-2070	50 mL, clear glass
Guard column	Agilent Technologies	959757-902	Zorbax Eclipse Plus-C18 Narrow Bore Guard Column (2.1 x 12.5 mm, 5 µm)
Hydrochloric acid	Merck	1.00317.1000	
L-kynurenine	Sigma-Aldrich	K8625	≥98% (HPLC)
Magnetic stirrer	Wigo		ES 21 H
Microbalance	Mettler Toledo		model XP6
Milli-Q system	Millipore	ZRQSVPO30	Direct-Q 3 UV with Pump
Quadrupole mass spectrometer	Agilent Technologies	G1948B	model 6120
Penicillin-Streptomycin	Sigma-Adrich	048M4874V	
Plate reader	Bio Tek		Synergy 2 operated by Gen5
Potassium chloride	Merck	1.04936.1000	
Potassium dihydrogen phosphate	Merck	1.05108.0500	
Protein Assay Dye Reagent Concentrate	BioRad	500-0006	
See-saw rocker	Stuart	SSL4	
Serological pipette	Nest	326001	5 mL, polystyrene, non-pyrogenic, sterile
Sodium chloride	Sigma-Aldrich	7647-14-5	
Sodium phosphate dibasic	Merck	1.06346.1000	
Solvent inlet filter	Agilent Technologies	5041-2168	glass filter, 20 µm pore size
Solvent reservoir (for LC-MS)	Agilent Technologies	9301-6524	1 L, clear glass
Solvent reservoir (for LC-MS)	Agilent Technologies	9301-6526	1 L, amber glass
Spreadsheet program	Microsoft Office		Microsoft Office Excel
Stir bar	Bionovo	6-2003	teflon coated
Syringe filters for culture medium filtration	Bionovo	7-8803	regenerated cellulose, Ø 30 mm, 0,45 µm
Syringe filters for mobile phase components filtration	Bionovo	6-0018	nylon, Ø 30 mm, 0,22 µm
Tissue culture plates	VWR	10062-900	96-wells, sterille
Trypsin-EDTA Solution	Sigma-Aldrih	T4049-100 mL	
Ultra-High Performance Liquid Chromatography system	Agilent Technologies	G1367D, G1379B, G1312B, G1316C	1200 Infinity system consisted of autosampler (G1367D), degasser (G1379B), binary pump (G1312B), column thermostat (G1316C)
Ultrasound bath	Polsonic	104533	model 6D
Vortex	Biosan		model V-1 plus
Xanthurenic acid	Sigma-Aldrich	D120804	96%

Rebuttal Letter
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JoVE61031R1 "Simultaneous Quantification of Selected Kynurenines Analyzed by Liquid Chromatography-Mass Spectrometry in Medium Collected from Cancer Cell Cultures "

We thank all Reviewers for their constructive comments. Our changes within the revised manuscript are marked in red.

Editorial comments:

1. Please copy-edit the manuscript as the language is not publication grade.

The manuscript has been again reviewed very carefully to address all grammatical errors. In addition, an experienced scholarly writer edited the manuscript. The major changes are marked in red.

2. Please revise the highlighting to be 2.75 pages or less. Please highlight the entire steps to ensure that a complete and coherent story is told. We need to know the actions and the details required to perform that action in order to film.

The action has been highlighted in yellow and constitutes no more than 2.75 pages.

3. Please address all the specific comments marked in the manuscript. Please use the attached version for revision. This is formatted to match the journal's style.

All comments have been addressed.

4. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in Figure Legend, i.e. “This figure has been modified from [citation].”

We did not copy the same results that are published in our JPBA paper, but used the chromatogram of the other repeat, despite that the chromatogram looks very similar. In addition we have added citation for the original JPBA paper.

Reviewers' comments:

Reviewer #3:

Minor Concerns:

There is no comparison of this method with the previous established methods, in particular liquid chromatography with ultraviolet or fluorescent detection. A comparison of peaks obtained via the current described methods from MDA-MB-231 cells and SK-OV-3 cells, with peaks obtained using these prior methods, and a brief description of the observations (what are discrepancies? how does the peak quality compare?) would help strengthen the manuscript.

Answering this question, we would like to emphasize that it was not our goal in this paper to compare different methodologies that can be employed for kynurenine (Kyn) quantification. Similarly, the fluorescence-based quantification was not a subject of our work. However, in our other studies, we have employed liquid chromatography coupled with ultraviolet detection (DAD as detector) for Kyn determination. Unfortunately, comparison of the results obtained using LC-MS and HPLC-DAD is hampered by several methodological discrepancies like gradient programs, applied mobile phases, retention times of the same metabolites, as well as difficulties to separate some interfering co-eluting compounds present in the material derived from living cells. In addition to other studies, our recently published results (Sadok et al., Simultaneous voltammetric analysis of tryptophan and kynurenine in culture medium from human cancer cells, *Talanta* 209 (2020) 120574, DOI: 10.1016/j.talanta.2019.120574) show that LC-MS allows for detection and quantification of lower amounts of Kyn in comparison to LC-DAD method. Determination of Kyn generated by MDA-MB-231 cells was difficult by LC-DAD (detected, but under LOQ), whereas LC-MS was suitable for quantification of the same metabolite in medium from cultured cells. Other metabolites (3-hydroxykynurenine, 3-hydroxyantrahilic acid, xanthurenic acid) were not thoroughly studied in cell cultures using LC-DAD thus we do not have enough data to comment on this topic.

Reviewer #4:

It seems that this version of the manuscript is a revised version of a previous one (?) but it was difficult to understand which changes and improvements have been made.

Correct, this is a revised version of the manuscript. The changes introduced in response to previous reviewer's comments have been marked in red in revised version.

Major Concerns:

1. the internal standard used, 3-nitrotyrosine (3NT), is -as stated by the authors- the main limitation of this protocol. Tyrosine nitration is a chemical modification that occurrs during oxidative stress conditions. Certain types of cancers induce oxidative stress and thus 3-NT formation, although it is not clear to what extent. The use of 3-NT as the internal standard limits the flexibility of the presented protocol and potentially introduces a bias in the quantification of the analytes. I agree that the use of ILIS is not always necessary, although it represents the best internal standard in MS detection. However, the authors could (and should) have selected other commercially available molecules that have similar chromatographic properties and are not produced endogenously by mammalian cells.

During method development we have examined the tested samples (medium collected after culturing cancer cells (MDA-MB-231 and SK-OV-3) for presence of the endogenous 3-NT. There was no detected trace of this compound. Furthermore, endogenous 3-NT is present in cell culture medium at negligible amount (Teixeira et al., Development of a new HPLC-based method for 3-nitrotyrosine quantification in different biological matrices, Journal of Chromatography B 1046 (2017) 48-57, DOI: 10.1016/j.jchromb.2017.01.035)
compared to the exogenously added 3-NT thus it will have no impact on quantification. We emphasized in line 611 of the manuscript that endogenous 3-NT level should be initially verified. In our future path, we will consider testing other types of internal standards.

2. This manuscript represents the "step-by-step" version of a protocol published elsewhere by the same authors. However, the protocol here seems to be a simplified version of the published one, which is odd considering the scope of JoVE. I would strongly encourage the authors to describe the entire protocol providing more details. For general interest, it would be better to include a short explanations of why a certain reagent or procedure was chosen (e.g. different extraction protocols, protein precipitation procedures, LC buffers and so on). Moreover, there is limited information regarding the cell culture procedures, including duration of incubation, need for LC-SQ data normalization (e.g. determination of protein content), etc.

During the method development each step of the LC-MS analysis was carefully optimized and is described in our previous paper (Sadok et al., Application of the optimized and validated LC-

MS method for simultaneous quantification of tryptophan metabolites in culture medium from cancer cell, *Journal of Pharmaceutical and Biomedical Analysis* 176 (2019) 112805). The rationale for selecting the critical conditions of analysis (LC buffers, reagent for protein precipitation) has been also explained there. Instead of copying the previous article, our goal was to describe handy protocol of kynurenines determination in culture medium from cancer cells. Thus, inhere we focused on the practical aspects providing some tips and recommendations how to perform the analysis. Considering the Reviewer's suggestion, we decided to add to the protocol a description of cell culture conditions, protein determination and data normalization (point 7 and 9 in the protocol). In addition, a brief summary on selection of solvent for protein removal and LC mobile phase composition have been included (DISCUSSION, paragraph 3).

3. There is no explanation of why charcoal treatment is performed. I assume it is done to remove tryptophan (Trp) and its catabolites from the culture medium used (please, specify). Removing Trp from the culture media represents a big challenge for any kind of mammalian cell, and, among other things, will modify the whole kynurenine pathway activity. It is likely that this protocol will be used to study the activity of the kynurenine pathway in different cells types or how it is modulated by different challenges/treatments. To this end, it would be more suitable to use a Trp-containing media. Charcoal treatment could be applied to FBS, which potentially introduces variable amounts of kynurenines, or FBS could be replaced with BSA (mostly for short incubations).

The charcoal treatment was used only to prepare the calibration standards and quality control samples. The experimental samples were not treated with charcoal. Charcoal treatment was applied to remove the tryptophan catabolite - kynurenine (Kyn) as it was found in the initial culture medium (DMEM-HG supplemented with glucose and FBS) and could impact proper calibration. The second paragraph of the 'REPRESENTATIVE RESULTS' section discuses this issue. We have also provided Figure 1, which compares results obtained for the complete and charcoal treated culture medium. Definitely, during charcoal treatment the amount of other compounds like tryptophan are significantly reduced or removed. Nevertheless, as it was highlighted, charcoal purification was used only for preparation of calibration curves. In case of experimental samples, the complete medium (without purification) used for cell culturing was also analyzed, and the determined Kyn was subtracted from the amount measured in

medium collected from the cultured cells. This information can be found in the 'REPRESENTATIVE RESULTS' section (at the end of the second paragraph).

The authors detect an unknown peak that is probably Trp (fig 3) but it is unclear how they concluded that this peak corresponds to Trp? The retention time of compound X (Fig 3) and Trp (Fig 4) seem different enough to suggest that these two are different molecules. In addition, the retention time of XA, present in both figures, is more or less the same, suggesting that the retention shift between compound X and Trp is not due to an LC issue. It is also surprising that the concentration of Trp is so much higher in the medium of one cell line vs the other? Is the assumption that this is all due to protein catabolism occurring in MDA-MB-231 cells? In figure 1 you show the analysis of the medium used before and after charcoal treatment, but there is no trace of Trp (or compound X, if they correspond). How can this be explained? A standard DMEM medium contains more than 10 mg/L of Trp. This point should be clarified. I would recommend including Trp in this analysis since its standard is easily accessible and inexpensive. Moreover, this would give the possibility to draw some conclusions regarding IDO/TDO activity (as suggested by the authors in the conclusions section).

This is correct that MDA-MB-231 and SK-OV-3 cells utilize Trp at different pace. It was confirmed in our other work (Sadok et al., Simultaneous voltammetric analysis of tryptophan and kynurenine in culture medium from human cancer cells, *Talanta* 209 (2020) 120574, https://doi.org/10.1016/j.talanta.2019.120574), where we attempted to determine Trp and Kyn content in medium from MDA-MB-231 and SK-OV-3 cultured cells. We found that MDA-MB-231 utilizes Trp in smaller extent compared to SK-OV-3 cells resulting in lower concentration of Kyn. Furthermore, our observations of standard DMEM using LC-DAD analysis (unpublished data) always showed the signal from Trp. It is not surprising, since Trp is one of the culture medium ingredients. In the present paper employing LC-MS to study tryptophan metabolism via kynurenine pathway the LC-MS system was set to monitor ions selected for individual metabolites at the defined timeframe. Thus, Trp could not be observed on chromatogram since we did not include in LC-MS settings the ions unique for Trp. We verified once again the LC-MS results obtained during DMEM analysis from different experiments. Below we present in Fig. R1 another example of chromatogram of DMEM prepared according to herein described LC-MS protocol (untreated with charcoal, spiked with 3NT). We do not see a significant peak that might correspond to Trp (probably it is caused by

apparatus settings that in this case did not allow for recording of the characteristic for Trp ions). We agree with Reviewer that we jumped into conclusions about compound X too fast. We removed that assumption from the text since it is not clear what the compound X is. This issue will be interesting to dwell on in the future, however it is not a subject of this paper.

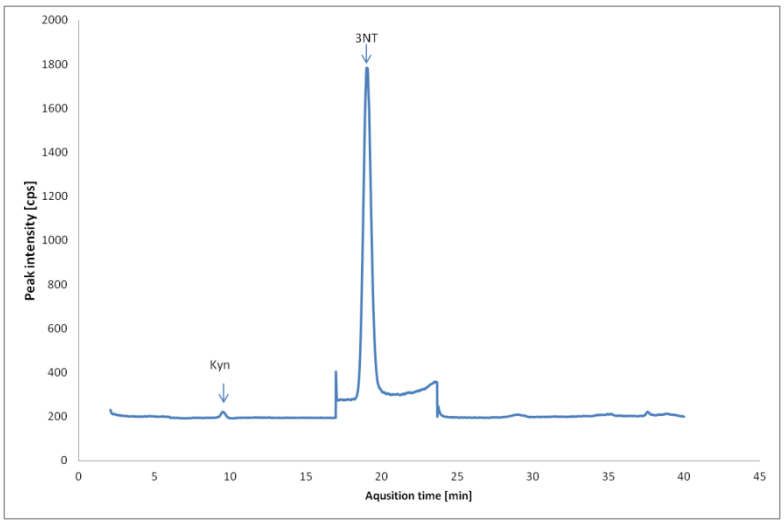

Fig.R1. Example of LC-MS result obtained during analysis of complete DMEM used for MDA-MB-231 and SK-OV-3 culture spiked with 3NT (untreated with charcoal).

Since Trp was not included in our protocol it is not possible to add this compound to our analysis without extensive methodological adjustments. Simultaneous analysis of several compounds is more complicated than analysis of just one analyte. The addition of this analyte will require re-adjustment of the method settings including evaluation of Trp ionization in the selected mobile phase or assessment of different mobile phase for simultaneous analysis of 3HKyn, Kyn, 3HAA, XA, Trp and 3NT (as internal standard). Also sample preparation step might differ, e.g. significant loss of Trp might be observed during protein precipitation using the selected solvent for 3HKyn, Kyn, 3HAA, XA. Moreover, the final protocol (after Trp addition) should be validated in terms of linearity, precision, accuracy, and recovery to assess whether the method provides reliable results. It might be the goal of a new project to expand on additional analytes.

4. Figure 2A looks rather similar to figure 3 in ref. 22. Is this in line with JoVE's publication policies? Otherwise permission from the ref 22 journal should be obtained.

All figures prepared for the JoVE were not used elsewhere, despite a fact they might look very similar. However, to comply with the JoVE policy we included the citation in the figure legend along with a statement that the figure has been modified from Sadok et al [27].

Minor Concerns:

1. There are some steps of the protocol that are too specific to a given instrument or lab setting (e.g. automatic pipette, evaporator, use of an autosampler, …). Unless these points/instruments are important (in this case it should be specified), this should be re-write it in a more general way. On the other side, it is very useful to have your UHPLC-SQ set up, calibration curve concentrations and so on.

The details were provided in response to the previous review comments in order to describe the action and show how critical steps are performed. It is indicated when a reader should follow the specific instrument manual.
The other mentioned details on calibration and so on are provided in the cited paper [27] and intentionally are not repeated in here.

2. In the protocol section, subheadings could be differentiated from the text a bit more clearly. For instance, subheading 1: "prepare the standard stock solutions of …." Could be replaced with "stock solutions of 3NT, Kyn, 3HKyn, 3HAA, XA" or something similar.

Appropriate changes have been introduced to the protocol section.

3. The overall text should be improved in terms of style, punctuation, and choice of words as understanding what the authors mean is sometimes difficult. JoVE seems to offer copy editing services. If so they should be used.

The manuscript has been edited by an experienced scholarly writer.

4. In the discussion section, certain considerations should be restricted to information what the data from this protocol can provide. The Kyn pathway includes many metabolites, some of them with direct and indirect effects on the levels of other, so the present analysis can only go so far in terms of providing a complete picture of the pathway.

We agree and did not aim to extensively draw conclusions from the results on determined Kyn levels. Rather we show possible directions in studying regulation of Kyn pathway by measuring

changes in concentration of several metabolites (instead of Kyn only) that is possible using the presented protocol.

Minor - detailed:

1. Title: …culture medium FROM cancer cell… sounds like that the media was produced by the cancer cells.

Title has been changed and now it reads:

"Simultaneous Quantification of Selected Kynurenines analyzed by Liquid Chromatography-Mass Spectrometry in medium collected from Cancer Cell Cultures"

2. Summary, line 24: "four kynurenine pathway tryptophan metabolites" is not grammatically correct and is a confusing sentence.

The sentence now reads:

Described here is an accessible protocol for determination of four different tryptophan metabolites generated in kynurenine pathway….

3. Abstract, line 30: tryptophan derived metabolites (and in line 33, tryptophan derived products) are generally termed as tryptophan catabolites.

Corrected.

4. Abstract, line 35: "reached" is probably not the right word. Moreover, the medium is mostly analyzed because it reflects some of the metabolic events that occur at the cellular level.

The sentence was corrected accordingly.

5. Introduction, lines 59-60: description of the kynurenine pathway could be expanded and the reader would benefit from a schematic recap of it.

The description of kynurenine pathway was expanded according to the suggestion.

6. Introduction, line 62: XA is not mentioned in ref. 1

Additional references have been added.

7. Introduction, lines 68-69: sentence: "Some changes in Trp metabolism reveal in the tumor microenvironment and cancer cells" is not clear…

Corrected and it reads: "Some changes in Trp metabolism are observed in tumor microenvironment and cancer cells[2, 8]."

8. Introduction, lines 69-73: there is the need to have good methods to simultaneously detect as many kyn metabolites as possible from different matrixes, with easy, flexible, and reliable protocols.

We changed this paragraph based on the suggestion and it now reads:

"Therefore, there is the need to have appropriate methods to simultaneously detect as many KP metabolites as possible in variety of biological specimens, with easy, flexible, and reliable protocol."

9. Introduction, line 77: in my opinion, it is inappropriate to have ref. 7 there. Moreover, the whole sentence has to be rewritten. Lastly, the analysis done using commercially available kit could be very fast.

We added the original reference by Takikawa et al that can educate readers on the colorimetric assay (p-dimethylaminobenzaldehyde - Ehlirich reagent) for kynurenines quantification. It is non-specific and several compounds might interfere with Kyn therefore in modern analysis the chromatographic methods are preferred. The sentence has been also reworded for clarity.

c10. Introduction, lines 85-90: too much emphasis on the analysis of the cell culture medium. It is important, of course, but it is not an absolute novelty.

We edited this paragraph to express the intention to be more general for in vitro assays.

11. Introduction, lines 91-94: a robust protocol should work well for most currently available cell culture media. It is more relevant to have protocols that include as many kyn metabolites as possible.

Similar to comment #10.

12. Protocol, point 1.1: 0.3 mg is a very little amount of compound. Weighing this amount is risky since it could introduce a huge error in the analyte quantification.

We used in our laboratory a precise microbalance to weight reagents and analytical grade standards are usually expensive owing their instability in solutions. This is only an example how to prepare standards. Other users might use higher amounts to prepare standards keeping the given proportions. This suggestion has been added to the protocol.

13. Protocol, points 1.2s: can be very easily condensed and better phrased.

Corrected.

14. Protocol, point 1.2.4, NOTE: why three cycles?

It is recommended because of standards' instability. The explanation has been added.

15. Protocol, point 2.5: does "cold" methanol mean ice cold or kept at -20C?

We assume the comment relates to step 3.2. Methanol was kept at -20C. This information was added.

16. Protocol, point 3.8: a cloudy sample could indicate that there is some protein left-over or that the compound is not properly solubilized.

Correct, we added this explanation.

17. Protocol, point 5.5: I would recommend to ensemble the guard column before purging and flushing the system.

Corrected.

18. Protocol, point 5.6.1: this gradient is very long considering the type of detection that you are using. Could it be improved? Alternatively, could it host other Kyn metabolites?

In this protocol we used Single Quadrupole (SQ) mass detector that is less selective compared to Triple Quadrupole, because it does not contain collision cell and is not able to fragment ions. Thus, when working with SQ, the detection and analytes identification is based on both retention time and monitoring of selected ions characteristic for the target compounds. Some

sample components might generate ions with m/z selected for the analyte. In our opinion, it is better to apply lower gradient to obtain better peak separation and reduce errors associated with interfering matrix components. In such settings the protocol can be further expanded onto other compounds. It might require some adjusting in gradient program.

19. Protocol, point 6: bolt

Corrected

20. Protocol, point 6.2: redundant

The point 6.2 was removed according to the suggestion.

21. Protocol, point 6.4.2: if you do repeat charcoal purification, the medium should be cleared from your interfering analytes. Otherwise, repeat treatment one more time or do not apply any charcoal purification and consider the amount of these molecules before and after incubation with the cells.

The suggested clarification is included in point 2.5.

22. Protocol, point 6.4.3.1-2: explain it better.

Some explanation has been added.

23. Protocol, 6.4.4 NOTE: this is not correct, intraday and interday variability should be checked carefully.

The NOTE has been removed from the text.

24. Protocol, point 7.1 NOTE: if you want to compare the medium from different wells (containing the same cell type ± treatments) you should make sure that the same number of cells per well is seeded and the same number of cells is considered during the experiment. Perhaps, specify that protein content analysis could normalize the results.

We agree with Reviewer. That information was included as a NOTE in step 7. We also added a description how to estimate protein content (answering previous recommendations) in protocol step 9.

25. Protocol, point 7.5 NOTE: alternatively, you can add more points in your calibration curve (if solubility and linearity are not a problem) to adjust it to your sample concentration. Moreover, "diluted matrix" is a rather confusing definition, please, change it with a more appropriate word (this applies also in other parts of the text).

The phrase “Diluted matrix” has been changed to “100 times diluted culture medium” and the note expended with the recommended information.

26. Representative results, line 420: English, check it.

Corrected

27. Discussion: check typos (e.g. poropose, line 485, extremaly, line 525, etc), and proper terms (e.g. Kyn is the most profound Trp metabolite, what does it mean?)

Corrected.