

Submission ID #: 69558

Scriptwriter Name: Sulakshana Karkala

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Title: Analysis of Vitamin A Stable Isotope Tracers using LC-MS/MS

Authors and Affiliations:

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Author Questionnaire

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

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

Videographer: Please film the instrument's screen for all shots labelled as SCREEN

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

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes**

Current Protocol Length

Number of Steps: 32

Number of Shots: 61

Introduction

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

NOTE: For the interview audio, one channel was captured at a lower level than the other to help accommodate any sound peaking. I recommend using only the louder channel as mono. All headshots of the authors for both projects were uploaded to the folder for Shoot 69558.

INTRODUCTION:

~~What is the scope of your research? What questions are you trying to answer?~~

- 1.1. **Georg Lietz:** My research focuses on high-throughput quantification of native and isotope-labeled vitamin A in human plasma to accurately assess total body vitamin A stores.
 - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

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

- 1.2. **Georg Lietz:** Recent advances in high-sensitivity LC–MS/MS allow lower isotope dosing, faster analysis, simplified workflows, and scalable application of vitamin A isotope dilution studie
 - 1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~What are the current experimental challenges?~~

- 1.3. **Georg Lietz:** Current challenges include maintaining tracer stability, preventing isomerization, ensuring precise dosing, and achieving sufficient analytical sensitivity at low isotope concentrations.
 - 1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What significant findings have you established in your field?~~

- 1.4. **Georg Lietz:** We developed and validated RID dosing and LC–MS/MS methods that enable accurate measurement of total body vitamin A across diverse populations.

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

~~What questions will future research focus on?~~

- 1.5. **Georg Lietz**: Standardization uses harmonized protocols, reference materials, and guidelines, while quality assurance ensures reproducibility, consistency, and cross-study comparability.

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

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

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

NOTE: Title was not provided

What problem in your field of research does your JoVE article address?

1.6. **Georg Lietz**: (authors will present their testimonial statements live)

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

How does the video format of your article help you to share your work with the broader scientific community?

1.7. **Georg Lietz**: (authors will present their testimonial statements live)

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

Ethics Title Card

This research has been approved by the Research Ethics Board at the University of the Philippines

Protocol

2. Preparation of Calibration Standards and Internal Standard Control Samples

Demonstrators: Alice Goddard and Georg Lietz

2.1. To begin, obtain an internal standard stock solution of retinyl acetate in ethanol [1]. Dilute the stock standard from 1 milligram per milliliter to 1 microgram per milliliter [2].

2.1.1. WIDE: Talent holding labeled stock solution.

2.1.2. Shot of a labelled working standard volumetric flask.

2.2. Using a pipette, dilute 60 microliters of the stock standard with 300 microliters of ethanol [1], then dilute 60 microliters of this solution in 10,000 microliters of ethanol [2]. ~~Add 20 microliters of the working internal standard solution to each sample, corresponding to 20 nanograms of retinyl acetate [3].~~

2.2.1. Talent pipetting stock standard into ethanol to prepare the first dilution.

2.2.2. Talent performing the second dilution with ethanol.

~~2.2.3. Talent adding the internal standard working solution to sample tubes.~~ NOTE: Redundant information combined with 2.8 and 2.9

2.3. Prepare the calibration standard working solution by diluting 100 microliters of the calibration stock standard with 10,000 microliters of ethanol in a volumetric flask [1]. Measure the absorbance of the diluted calibration stock solution at 325 nanometers using a spectrophotometer with a quartz cuvette to determine its concentration [2].

2.3.1. Talent pipetting calibration stock standard into a volumetric flask containing ethanol. NOTE: For shot 2.3.1, an additional shot was required and has been marked as 2.3.1 1B.

2.3.2. Talent measuring absorbance at 325 nanometers using a spectrophotometer.

2.4. Next, take an aliquot of the diluted tracee calibration stock solution [1] and adjust the concentration to 8 micromolar using a mixture of 50 percent ethanol and 50 percent acetonitrile-deionized water [2-TXT].

2.4.1. Talent pipetting out the diluted tracee calibration stock solution. NOTE: 2.4.1 and 2.4.2 are filmed in a single shot

2.4.2. Talent preparing tracee calibration solutions using mixed solvents.

2.5. Take an aliquot of the diluted tracer calibration stock solution [1] and adjust the concentration to 2 micromolar using 100 percent ethanol [2-TXT].

2.5.1. Talent pipetting out the diluted tracer calibration stock solution. NOTE: Shots 2.5.1 and 2.5.2 were recorded as 2.4.1 take 1.

2.5.2. Talent preparing tracer calibration solutions using 100 percent ethanol.

2.6. Prepare duplicate calibration standards of the tracee using an equimolar solution of ethanol and acetonitrile-deionized water, ranging from 6 micromolar to 0.03 micromolar [1]. **NOTE: The VO is edited for the deleted shot**

~~2.6.1. Talent preparing multiple calibration standards in labeled tubes.~~ **NOTE: Not filmed**

2.6.2. Shot of labeled calibration standards showing concentration range.

2.7. Prepare duplicate tracer calibration standards in 100% ethanol, with concentrations ranging from 0.4 micromolar to 0.004 micromolar [2]. **NOTE: The VO is edited for the deleted shot**

~~2.7.1. Talent preparing multiple calibration standards in labeled tubes.~~ **NOTE: Not filmed**

2.7.2. Shot of labeled calibration standards showing concentration range.

2.8. For the preparation of internal standard control samples, first combine 120 microliters of ethanol with 200 microliters of acetonitrile-deionized water and vortex [1].

2.8.1. Talent pipetting 120 μL ethanol into a tube and adding 200 μL of acetonitrile/ dH_2O .

2.9. Pipette 20 microliters of the internal standard working solution into three separate 0.6-milliliter tubes [1]. Add 80 microliters of the ethanol-mobile phase mixture, then vortex to mix the suspension [2].

2.9.1. Talent pipetting 20 μL of the internal standard working solution into three separate 0.6-milliliter tubes.

2.9.2. Talent adding 8 μL Ethanol-mobile phase mixture into the tubes and vortexing it.

2.10. Transfer the internal standard control samples to amber HPLC vials with inserts [1] and immediately cap the vials to prevent solvent loss [2].

2.10.1. Talent transferring the controls into HPLC vials.

2.10.2. Shot of the vials being capped.

3. Extraction of Retinoids from Plasma

3.1. Remove plasma samples from a minus 80 degrees Celsius freezer [1] and allow them to defrost at room temperature for approximately 30 minutes [2].

3.1.1. Talent removing frozen plasma samples from the freezer. **NOTE: Shot 3.1.1 was marked with the wrong shoot number.**

3.1.2. Talent placing them on the bench.

- 3.2. Then, vortex each plasma sample [1]. After briefly spinning the sample to collect liquid from the lid, place the samples on ice [2].
 - 3.2.1. Talent placing the samples on a vortex machine.
 - 3.2.2. Talent places the sample on ice.
- 3.3. Turn on a refrigerated centrifuge and allow it to cool to 4 degrees Celsius [1]. Pipette 200 microliters of deionized water into labeled 15-milliliter glass tubes with screw caps [2].
 - 3.3.1. Talent setting centrifuge temperature to 4 degrees Celsius.
 - 3.3.2. Talent adding 200 μ L deionized water into the 15 mL labeled glass tubes.
- 3.4. Then pipette 20 microliters of the internal standard working solution into each glass tube [1]. Dispense 200 microliters of plasma into the corresponding tube containing internal standard and deionized water [2].
 - 3.4.1. Talent pipetting 20 μ L internal standard into glass tubes.
 - 3.4.2. Talent adding 200 μ L plasma samples to prepared glass tubes.
- 3.5. Add 400 microliters of ethanol to each tube [1] and gently swirl to mix, avoiding vortexing to prevent protein adhesion to the glass [2].
 - 3.5.1. Talent adding 400 μ L ethanol to the tubes.
 - 3.5.2. Talent gently swirling tubes by hand.
- 3.6. For the first extraction, add 2 milliliters of hexane to each tube and tightly fasten the lids [1].
 - 3.6.1. Talent adding hexane and securing tube caps.
- 3.7. Place the tubes on an orbital shaker and shake at 15,000 rpm for 30 minutes [1]. Centrifuge the tubes in a refrigerated centrifuge at 18,000 *g* for 10 minutes at 4 degrees Celsius [2].
 - 3.7.1. Talent placing tubes on the orbital shaker.
 - 3.7.2. Talent loading tubes into the refrigerated centrifuge.
- 3.8. Next, using glass Pasteur pipettes, transfer the supernatant to clean, labeled glass tubes without disturbing the protein precipitate or aqueous phase [1].
 - 3.8.1. Talent carefully transferring the hexane supernatant with a Pasteur pipette.
- 3.9. Perform a second extraction by adding another 2 milliliters of hexane to the remaining residue and fastening the lids [1]. Shake at 15,000 rpm for 15 minutes, then centrifuge [2].
 - 3.9.1. Talent adding hexane for the second extraction.
 - 3.9.2. Shot of the tubes shaking in an orbital shaker.

- 3.10. Transfer the supernatant from the second extraction into the tube containing the first extraction supernatant [1]. Then evaporate the combined hexane extracts under a gentle stream of nitrogen in a darkened fume hood with the heating block set to 35 degrees Celsius [2].
 - 3.10.1. Talent combining supernatants using a Pasteur pipette.
 - 3.10.2. Talent placing tubes under nitrogen gas in the fume hood.
- 3.11. Resuspend the dried residue in 50 microliters of ethanol and vortex briefly [1]. Then add 50 microliters of mobile phase before vortexing again [2].
 - 3.11.1. Talent resuspending dried residue with ethanol.
 - 3.11.2. Talent adding 50 μ L of mobile phase.
- 3.12. Transfer the solution into labeled 0.5-milliliter microcentrifuge tubes [1] and centrifuge the suspension at 16,900 *g* for 5 minutes [1].
 - 3.12.1. Shot of solution being pipetted into a labeled 0.5 mL microcentrifuge tube.
 - 3.12.2. Talent placing the tubes in a centrifuge and setting the parameters.
- 3.13. Transfer 80 microliters of the reconstituted residue into amber HPLC vials fitted with 300-microliter inserts and immediately cap the vials [1].
 - 3.13.1. Talent transferring extract into amber HPLC vials and capping them.

4. Tuning of the LC-MS/MS for Retinol Quantification

Demonstrator: Dan Astley

- 4.1. Fill a one milliliter Luer-lock Hamilton syringe with infusion solution at 10 micrograms per milliliter and connect it to the mass spectrometer source using PEEK (*Peek*) tubing [1]. Set the infusion flow rate to 10 microliters per second [2].
 - 4.1.1. Shot of a 1 mL Luer-lock Hamilton syringe being filled with infusion solution and being connected to the mass spectrometer using PEEK tubing.
 - 4.1.2. Talent setting infusion flow rate on the syringe pump.
- 4.2. Select **Manual Tuning** and ensure the instrument is in **Tune and Calibrate Mode** [1]. Start the syringe pump and select **Q1 Scan** in positive ion mode [2].
 - 4.2.1. SCREEN: (First-take)-JoVE---69558---4-2.mp4: 00:09-00:20.
Videographer: Please film the instrument's screen for all shots labelled as SCREEN
 - 4.2.2. SCREEN:: (First-take)-JoVE---69558---4-2.mp4: 00:32-00:43.
- 4.3. Set the lower mass range to 50 daltons and the upper mass range to approximately 100 daltons above the molecular mass of the target compound [1]. Then set the cycles to 500, the scan rate to 200 daltons per second, and click **Start** to begin acquisition [2].

- 4.3.1. SCREEN: (First-take)-JoVE---69558---4-3-to-4-4-(1).mp4: 00:06-00:10
- 4.3.2. SCREEN: (First-take)-JoVE---69558---4-3-to-4-4-(1).mp4: 00:13-00:19
- 4.4. After signal stabilization, verify the presence of expected precursor ion peaks, including singly and doubly charged ions [1].
 - 4.4.1. SCREEN: (First-take)-JoVE---69558---4-3-to-4-4-(1).mp4: 00:27-00:33 and 13C10-ROH_Q1 scan(4-3 to 4-4).PNG *Video editor: Please show the image as inset*
- 4.5. For the product ion scan, set the start mass at a lower mass, and the Stop mass just above the Q1 mass, then set the Cycles to 5 and the collision energy to 10 [1]. Check for Q1 mass as the precursor ion. If no smaller fragments appear, increase the energy to 20 [2].
 - 4.5.1. SCREEN:(First-take)-JoVE---69558---4-5-to-4-6.mp4: 00:06-00:40 and 13C10-ROH_Fragments at 10 V of collision energy (4-5 to 4-6).PNG *Video editor: Please speed it up and show the image as inset*
 - 4.5.2. SCREEN: (First-take)-JoVE---69558---4-5-to-4-6.mp4: 01:05-01:15 and 13C10-ROH_Fragments at 20 V of collision energy (4-5 to 4-6).PNG *Video editor: Please show the image as inset*
- 4.6. Adjust collision energy incrementally to generate product ions and observe secondary fragmentation [1].
 - 4.6.1. SCREEN:(First-take)-JoVE---69558---4-5-to-4-6.mp4: 01:30-02:00 and 13C10-ROH_Fragments at 30 V of collision energy (4-5 to 4-6).PNG *Video editor: Please show the image as inset*
- 4.7. ~~From the MS2 scan, select the three most abundant product ions for further optimization [1].~~ **NOTE: The information for the deleted shot is added in the next step**
 - 4.7.1. ~~SCREEN: (First-take)-JoVE---69558---4-7-to-4-9.mp4:~~ **NOTE: Not provided**
- 4.8. From the MS2 scan, select the three most abundant product ions for optimization. Enter the Q1 precursor and the Q3 product ion masses for each transition [1] and set the dwell time to 200 milliseconds, assigning descriptive names to each fragment [2].
 - 4.8.1. SCREEN: (First-take)-JoVE---69558---4-7-to-4-9.mp4: 00:07-00:17 and 13C10-ROH_CE optimisation (4-7 to 4-9).PNG *Video editor: Please show the image as inset*
 - 4.8.2. SCREEN: (First-take)-JoVE---69558---4-7-to-4-9.mp4 00:17-00:30 and 13C10-ROH_CE optimisation (4-7 to 4-9).PNG *Video editor: Please show the image as inset*
- 4.9. Choose collision energy and identify the optimal collision energy using the ramp feature [1]. Populate the collision energy column with the optimized value for each fragment

[2].

4.9.1. SCREEN: (First-take)-JoVE---69558---4-7-to-4-9.mp4: 00:37-00:56 and 13C10-ROH_CE optimisation (4-7 to 4-9).PNG *Video editor: Please show the image as inset*

4.9.2. SCREEN: (First-take)-JoVE---69558---4-10.mp4: 00:04-00:12

4.10. Refine declustering potential to minimize isobaric influences [1], then optimize collision cell exit potential for one transition at a time [2].

4.10.1. SCREEN: (First-take)-JoVE---69558---4-10.mp4: 00:17-00:29 and 01:30-01:33 13C10-ROH_CXP optimisation (4-10) *Video editor: Please show the image as inset*

4.10.2. SCREEN: (First-take)-JoVE---69558---4-10.mp4: 01:47-01:52 and 13C10-ROH_DP optimisation (4-10) *Video editor: Please show the image as inset*

4.11. Perform chromatographic separation on a reversed-phase column maintained at 40 to 50 degrees Celsius [1-TXT]. Inject 10 microliters of plasma extract and elute analytes at a flow rate of 0.4 milliliters per minute using the specified linear gradient [2].

4.11.1. Shot of LC setup.

4.11.2. SCREEN: (First-take)-JoVE---69558---4-11-2.mp4: 00:15-00:35

AND

TEXT ON PLAIN BACKGROUND:

For UPLC systems, linear gradient depends on research question

For only retinol determination, use

40% B to 60% B in 1.0 min

60% B to 95% B from 1.0 min to 6.0 min

100% B from 6.5 min to 7.5 min

100% B to 40% B from 7.5 to 7.6 min

40% B from 7.6 to 8.5 min

Video Editor: Please play both shots side by side in a split screen

4.12. After performing MS/MS (M-S-M-S) analysis, quantify retinol sample concentrations against their respective calibration standards.

4.12.1. SCREEN: (First-take)-JoVE---69558---4-12.mp4: 00:45-01:00

Results

5. Results

- 5.1. Based on the background noise observed in the m/z (*Mass-to-charge-ratio*) 279 to 100 transition [1], the level of detection and level of quantitation for retinol were established at 150 counts per second and 500 counts per second, respectively, corresponding to 6 femtomoles and 19 femtomoles on column [2].
 - 5.1.1. LAB MEDIA: Figure 1. *Video editor: Highlight 1A*
 - 5.1.2. LAB MEDIA: Figure 1. *Video editor: Highlight the dashed horizontal lines labeled LOD and LOQ in 1B*
- 5.2. Baseline chromatographic separation was achieved between native retinol and the non-labeled internal standard retinyl acetate, eliminating the need for a stable isotope-labeled retinyl acetate internal standard [1].
 - 5.2.1. LAB MEDIA: Figure 1. *Video editor: Highlight the two separate peaks corresponding to retinol and retinyl acetate in A and B*
- 5.3. Using UHPLC with a QTRAP 5500 system, a faster separation method achieved baseline resolution between retinol and retinyl acetate with retention times of 4.37 and 5.33 minutes [1].
 - 5.3.1. LAB MEDIA: Figure 3 A,B.
- 5.4. Native and isotope-labeled retinol together with native and labeled retinyl esters were separated within an 8.5-minute run, despite close retention times between retinol and retinyl acetate [1]. Under the faster UHPLC conditions, the close retention times of retinol at 2.10 minutes and retinyl acetate at 2.43 minutes required the use of isotopically labeled retinyl acetate to ensure accurate quantification [2].
 - 5.4.1. LAB MEDIA: Figure 4A
 - 5.4.2. LAB MEDIA: Figure 4. *Video editor: Sequentially show B and then C*

1. Retinyl acetate
Pronunciation link: <https://www.merriam-webster.com/medical/retinyl>
IPA: /'reʃ.ɪ.nəl 'æs.ə.tet/
Phonetic Spelling: ret·ih·nul ass·uh·tayt
2. Volumetric
Pronunciation link: <https://www.merriam-webster.com/dictionary/volumetric>
IPA: /,vɑː.ljə'met.rɪk/
Phonetic Spelling: vol·yuh·met·rik
3. Spectrophotometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/spectrophotometer>
IPA: /,spek.troʊ.fə'tɑː.mə.tə/
Phonetic Spelling: spek·troh·fuh·tom·uh·ter
4. Acetonitrile
Pronunciation link: <https://www.merriam-webster.com/dictionary/acetonitrile>
IPA: /ə'siː.tuː'naɪ.trɪl/
Phonetic Spelling: uh·see·toh·ny·tril
5. Equimolar
Pronunciation link: <https://www.merriam-webster.com/dictionary/equimolar>
IPA: /,iː.kwi'moʊ.lər/
Phonetic Spelling: ee·kwee·moh·ler
6. HPLC
Pronunciation link: <https://www.howtopronounce.com/hplc>
IPA: /,ertʃ.piː.el'siː/
Phonetic Spelling: aych·pee·el·see
7. Retinoids
Pronunciation link: <https://www.merriam-webster.com/dictionary/retinoid>
IPA: /'reʃ.ɪ.nɔɪd/
Phonetic Spelling: ret·ih·noyd
8. Hexane
Pronunciation link: <https://www.merriam-webster.com/dictionary/hexane>
IPA: /'hek.sen/
Phonetic Spelling: hek·sayn
9. Precipitate
Pronunciation link: <https://www.merriam-webster.com/dictionary/precipitate>
IPA: /prɪ'sɪp.ə.tət/ (noun), /prɪ'sɪp.ə.tet/ (verb)
Phonetic Spelling: prih·sip·uh·tuht ; prih·sip·uh·tayt
10. Reconstituted
Pronunciation link: <https://www.merriam-webster.com/dictionary/reconstitute>
IPA: /,riː'kɑːn.stə'tuː.tɪd/
Phonetic Spelling: ree·kon·stuh·too·tid
11. Luer-lock
Pronunciation link: <https://www.howtopronounce.com/luer-lock>
IPA: /'luː.ə lɑːk/
Phonetic Spelling: loo·er lok

12. Hamilton syringe
Pronunciation link: <https://www.hamiltoncompany.com/>
IPA: /'hæm.əl.tən sə'rɪndʒ/
Phonetic Spelling: ham·uhl·tuhn suh·rinj
13. Declustering
Pronunciation link: No confirmed link found
IPA: No verified IPA available
Phonetic Spelling: dee·klus·ter·ing
14. Isobaric
Pronunciation link: <https://www.merriam-webster.com/dictionary/isobaric>
IPA: / ˌaɪ.souˈbær.ɪk/
Phonetic Spelling: eye·soh·bar·ik
15. Chromatographic
Pronunciation link: <https://www.merriam-webster.com/dictionary/chromatographic>
IPA: / ˌkroʊ.mə.təˈɡræf.ɪk/
Phonetic Spelling: kroh·muh·tuh·graf·ik
16. Analytes
Pronunciation link: <https://www.merriam-webster.com/dictionary/analyte>
IPA: /'æn.əl.aɪt/
Phonetic Spelling: an·uh·lyte
17. Quantitation
Pronunciation link: <https://www.merriam-webster.com/dictionary/quantitation>
IPA: / ˌkwɑːn.təˈteɪʃən/
Phonetic Spelling: kwon·tuh·tay·shuhn
18. Femtomoles
Pronunciation link: <https://www.merriam-webster.com/dictionary/femtomole>
IPA: /'fem.toʊ.məʊlz/
Phonetic Spelling: fem·toh·mohlz
19. Isotopically
Pronunciation link: <https://www.merriam-webster.com/dictionary/isotopic>
IPA: / ˌaɪ.səˈtɑː.pɪ.kəl.i/
Phonetic Spelling: eye·suh·top·i·kuh·lee
20. UHPLC
Pronunciation link: <https://www.howtopronounce.com/uhplc>
IPA: / ˌjuː ˌeɪtʃ.pɪː.əlˈsiː/
Phonetic Spelling: yoo·aych·pee·el·see