tvst

Article

Quantifying Sample Collection and Processing Impacts on Fiber-Based Tear Fluid Chemical Analysis

Anis Barmada[1] **and Scott A. Shippy**[1,2]

[1] Department of Chemistry, University of Illinois at Chicago, Chicago, Illinois, United States
[2] Laboratory of Integrative Neuroscience, University of Illinois at Chicago, Chicago, Illinois, United States

Correspondence: Scott A. Shippy, Department of Chemistry, University of Illinois at Chicago, 845 West Taylor St, Chicago, IL 60607, USA. e-mail: sshippy@uic.edu

Received: February 5, 2020
Accepted: August 10, 2020
Published: September 23, 2020

Keywords: tear collection/analysis; sample processing; fiber; phenol red thread; ocular surface

Citation: Barmada A, Shippy SA. Quantifying sample collection and processing impacts on fiber-based tear fluid chemical analysis. Trans Vis Sci Tech. 2020;9(10):23, https://doi.org/10.1167/tvst.9.10.23

Purpose: Noninvasive analyses of tear fluid from humans and animal models in clinical and research settings most commonly use absorbent material for collection and processing. Still, the impact of these analytical techniques on tear chemical analyses remains largely unknown. The purpose of this study was to quantify the impacts of phenol red thread fiber-based tear sample collection and processing on the primary amine content.

Methods: Human tears were collected by placing the folded end of phenol red thread on the palpebral conjunctiva of the right eye for 20 seconds. The wetted thread was then processed using elution or extraction, and capillary electrophoresis with light-emitting diode-induced fluorescence detection was used for analysis and quantitation.

Results: Distinct processing methods impacted tear analysis differently. Primary amines adsorbed onto the thread partitioned in a chromatographic manner and thus any single portion of the wetted thread might not be representative of the whole sample. Quantitative assessment of five small molecule standards after on-thread processing showed significant overestimation of the actual concentration, with increased accuracy for larger volume samples. Yet collection of larger tear volumes introduced error in volume determination owing to evaporation and reduced small molecule separation resolution.

Conclusions: These results indicated that absorption-based tear fluid collection and processing significantly alter chemical content analysis, suggesting that the impacts of methods used should be regularly evaluated to standardize results drawn from different studies.

Translational Relevance: This study identifies potential inconsistencies and inaccuracies in tear analyses that are widespread across the published literature and clinical care.

Introduction

Tear film chemical composition, which includes electrolytes, proteins, lipids, and small molecule metabolites, is reflective of corneal biochemistry and physiology[1,2] and can ultimately be indicative of both ocular as well as systemic pathology.[3] This nature makes tear fluid analysis suitable for monitoring ocular surface health and disease.[4,5] The most commonly used methods for noninvasive tear analysis in both clinical and research settings from humans and animal models involve collection and processing via absorbent material, such as thread and filter paper.[6–17] Yet the impact of these analytical methods on the chemical composition of tear fluid is largely unexplored. It is crucial to define the impacts of collection and processing methods on tear analysis to standardize results drawn from different studies. To address this issue, we explored the impacts of phenol red thread fiber-based sample collection as well as processing on tear fluid chemical content using capillary electrophoresis with light-emitting diode-induced fluorescence detection.

Collection of tear fluid from humans and animal models for chemical analysis is typically performed either directly into a glass microcapillary tube or via an absorbent material.[18,19] The relatively large sampling volume associated with the capillary approach makes it less suitable for collection from rodents and aqueous tear-deficient patients. When tears are limited, a

washout of the tear film can be used, which facilitates collection but introduces an unknown dilution factor and impacts tear chemical analysis.[18,20,21] In addition, this approach requires extreme care and would likely require anesthesia with animal models, which has been reported to have significant impacts on the ocular surface.[22,23] Alternatively, the sorbent approach allows the collection of low-volume samples via capillary action and has been used previously with patients with dry eye[24] and in unanesthetized animal models.[14,16,17] Common absorbent material for tear collection include Schirmer filter paper strip, phenol red thread, and other cellulose ophthalmic sponges, with the Schirmer strip being used most often for tear analysis.[18] However, phenol red thread allows similar collection of tear fluid with a significantly smaller contact area, allowing lower tear sampling volume and minimal irritation and reflex impact on the ocular surface for subsequent chemical analysis.[14] Significant differences between tears collected by Schirmer strip and microcapillary tubes in metabolite,[25] lipid,[6] and protein[26] levels have been reported previously and suggested to originate from impacts of sample collection methods on tear analysis. Previous studies have investigated the impacts of Schirmer strip on tear proteins,[27–29] but both the impact of fiber threads or the influence on small molecules have not been previously studied. Because the tear processing method used is likely to impact tear analysis in a consistent manner, producing a systematic error, it is important to discover and define these impacts to standardize the results. To this end, we conducted an evaluation of the impacts of phenol red thread fiber-based tear sample processing on the tear primary amine content.

In the present study, we investigated the impacts of both thread-based sample collection and processing on tear fluid chemical analysis. In particular, we compared different tear processing methods and their impacts and assessed different regions of the wetted thread for absorbed analytes, as well as examining quantitation accuracy and electrophoretic separation resolution after on-thread processing with different sample volumes.

Methods

Chemicals and Reagents

Sodium phosphate, sodium hydroxide, and hydrochloric acid were purchased from Fisher Scientific (Itasca, IL). Amino acids, dimethyl sulfoxide, and sodium tetraborate decahydrate were obtained from Sigma-Aldrich (St. Louis, MO). Histamine dihydrochloride was obtained from Avocado Research Chemicals Ltd. (Heysham, UK). Potassium cyanide and 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) were supplied by Thermo Fisher Scientific (Waltham, MA). Ethanol (200 proof, USP) was obtained from Decon Laboratories, Inc. (King of Prussia, PA). Unless noted otherwise, solutions were prepared using 18.3 MΩ ultrafiltered, deionized water from US Filter Pure-lab Plus purification system (Lowell, MA). Phosphate buffer was prepared by dissolving anhydrous sodium phosphate dibasic in deionized water and adjusting the pH using 1 M hydrochloric acid. Borate buffer was prepared by dissolving sodium tetraborate decahydrate in deionized water at the desired concentration and adjusting the pH using 0.1 M sodium hydroxide. Amino acids and histamine were prepared in phosphate buffer (20 mM, pH 8.0). CBQCA was prepared in anhydrous dimethyl sulfoxide.

Tear Sample Collection

All human tear samples were collected from the right eye of the same subject without anesthesia using either glass capillary tubes or phenol red threads. For capillary-collected samples, a 1 µL Microcap glass capillary tube (Drummond, Broomall, PA) was inserted into the lower conjunctival sac. The thread-based tear sample collection method is based on our previous work.[14,15] Briefly, samples were collected via Zone-Quick phenol red threads (Showa Yakuhin Kako Co., Ltd., Tokyo, Japan; and Menicon America, Inc., Waltham, MA) by placing the folded end of the thread on the palpebral conjunctiva for approximately 20 seconds. Biological replicates were of the same volume and collected successively from the same subject. Phenol red threads that were previously used for the collection of human tears for educational purposes were procured for this study, and institutional review determined that this project did not meet the definition of human subject research. Nonetheless, informed consent was obtained with the tenets of the Declaration of Helsinki being followed.

Sample Processing

Capillary-collected tear samples were diluted in phosphate buffer before derivatization for comparison with thread-collected samples. The thread-based sample processing method is based on our previous work,[14,15] and the different methods compared in this study are depicted in Figure 1. In short, for tear samples, phenol red thread was air-dried after collection, the length of color change was measured

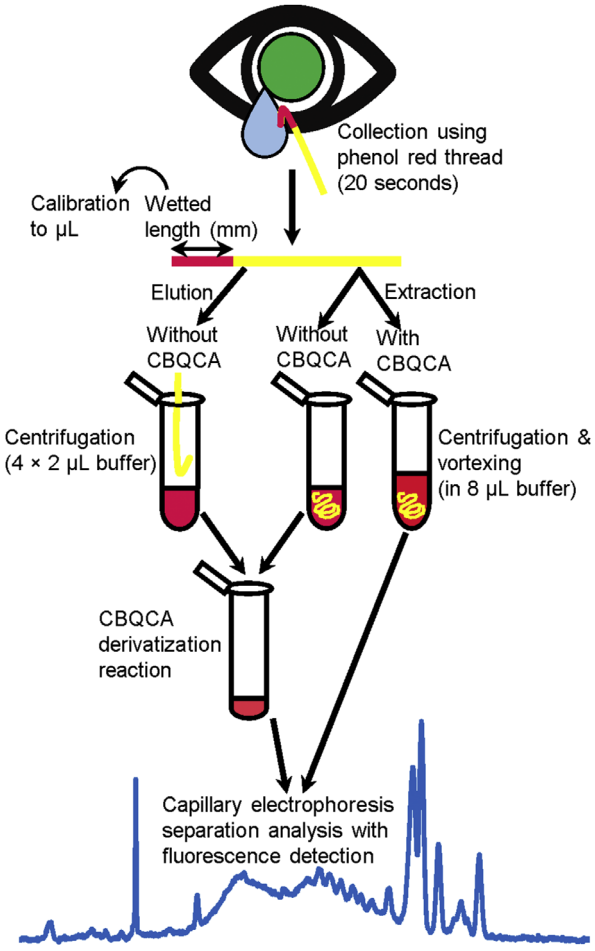

Figure 1. Schematic diagram illustrating the three different tear processing methods compared in this study. Tear fluid was collected by placing the folded end of a phenol red thread on the palpebral conjunctiva of the right eye for approximately 20 seconds, and the wetted length was measured. Tear samples were then processed through either elution off the thread with buffer then derivatization, extraction in buffer then derivatization, or a single-step extraction into the derivatization solution. Capillary electrophoresis with light-emitting diode-induced fluorescence detection was used for analysis and comparison.

using ImageJ software (National Institutes of Health, Bethesda, MD), and sample volume was determined using an external calibration curve ($y = 0.02x + 2.3$; $R^2 = 0.99$) as demonstrated previously.[15] For standards, samples were directly pipetted on the thread, the thread was air dried, and the known pipetted volume was used for calculations. Thereafter, for samples processed with elution, phenol red thread was inserted in a pipette tip placed in an Eppendorf microcentrifuge tube and then rinsed with phosphate buffer (4 × 2 µL; 20 mM; pH 8.0). The sample assembly was centrifuged (450×g for 5 minutes) to complete sample elution. For samples processed with extraction, the wetted region of the thread was cut using an ethanol-washed razor blade then vortexed (3200 rpm for 5 minutes) and centrifuged (2000×g) directly in phosphate buffer (8 µL). Thereafter, for samples collected via capillary tube or thread processed with elution and extraction, primary amines were labeled for fluorescence detection in a 1-hour reaction with equal volumes (1 µL each) of the sample, derivatization reagent CBQCA (10 mM), and potassium cyanide (10 mM). For thread-collected samples processed with a single extraction and derivatization step, CBQCA and potassium cyanide (1 µL each) were added directly to the vial containing the thread and phosphate buffer before centrifugation and vortexing. All derivatization reactions were performed in the dark to prevent photobleaching.

Capillary Electrophoresis

Electrophoretic separations were achieved using a laboratory-built capillary electrophoresis instrument with Zetalif detector (Picometrics, Paris, France) and light-emitting diode at 480 nm as described previously.[30] Separation conditions are based upon our previous work.[14] In short, the capillary was rinsed with 1 M sodium hydroxide, deionized water, and run buffer before analysis to obtain consistent separations. Gravimetric injections were used at a displacement of 15 cm for 10 seconds. All separations were carried out in a 50/360 µm inner/outer diameter capillary with a 34/44 cm effective/total length at 24 kV applied potential (546 V/cm field strength) using borate run buffer (20 mM; pH 9.2).

Data Analysis

Electrophoretic data were recorded via a custom Laboratory Virtual Instrument Engineering Workbench data acquisition software (National Instruments, Austin, TX). Electrophoretic peaks were identified via standard spiking. Peak heights were determined by subtracting the average baseline from the peak maximum. Percent normalized concentrations represent percent peak height obtained from the ratio of the peak height in the electropherogram of interest to the sum of heights of the same peak across all electropherograms from individual thread-collected samples. Relative fluorescence intensity per millimeter of phenol red thread was calculated by dividing the peak height by wetted thread length. External calibration curves were generated with standards for quantitation. Concentration values represent average concentration ± standard deviation. Microsoft Excel (Microsoft, Redmond, WA) was used to design figures and perform statistical analysis using the one-way

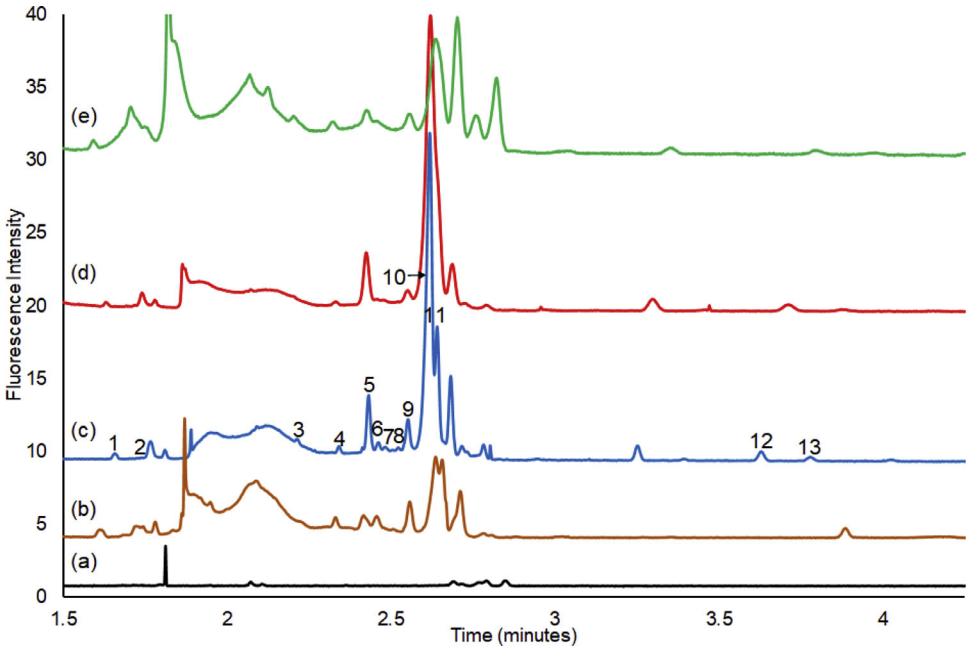

Figure 2. Comparison of human tear primary amines after sample processing with different methods of elution or extraction. Tear samples (0.6 µL) were collected from the same subject via phenol red threads or glass capillary tubes and derivatized with CBQCA for fluorescence detection by capillary electrophoresis. From the bottom up, the representative electropherograms correspond with (a) blank phenol red thread without tears, as well as tear samples collected via (b) glass capillary tube or thread processed by (c) elution (4 × 2 µL phosphate buffer) then derivatization, (d) extraction (8 µL phosphate buffer) then derivatization, and (e) single-step extraction and derivatization. The identified peaks correspond with (1) arginine, (2) histamine, (3) lysine, (4) citrulline/threonine, (5) glutamine, (6) asparagine, (7) methionine, (8) serine, (9) alanine, (10) taurine, (11) glycine, (12) glutamate, and (13) aspartate. The photomultiplier tube voltage was set at 600 V. Separation conditions: borate run buffer (20 mM, pH 9.2), 34/44 cm effective/total length capillary, and 546 V/cm field strength.

analysis of variance with repeated measures and two-tailed unpaired Student *t* test assuming unequal variances at the 95% confidence level. Unless noted otherwise, all experiments were repeated at least two times with similar results, and representative data were used.

Results

Impact of Tear Processing Method

In this experiment, we explored the impacts of phenol red thread-based tear sample processing on primary amine analysis. First, the impact of tear sample elution and extraction from thread was compared between three methods. Electrophoretic analyses of primary amines were performed with tear samples (1) eluted off the thread with buffer then derivatized, (2) extracted in buffer then derivatized, or (3) extracted directly in the derivatization solution. In addition, a blank thread without tears and glass capillary tube-collected tear sample were included for comparison.

The representative electropherograms in Figure 2 show that the blank phenol red thread itself does not produce appreciable background signal to interfere with tear contents. However, differences can be seen between tear samples collected via glass capillary tubes and threads, with a noticeably higher signal for the taurine peak of the thread samples compared with capillary-collected samples of similar volume. For thread-collected tear samples, processing with elution leads to higher separation resolution of primary amines compared with extraction variations. On average, elution produces taller and sharper peaks as shown by glutamine, alanine, taurine, glutamate, and aspartate peaks. In addition, asparagine, methionine, serine, and glycine peaks were mostly absent or unresolved in both samples processed with extraction. Several differences can also be seen between the two methods of extraction. Extraction followed by derivatization produces a higher separation resolution of primary amines and sharper peaks compared with a single-step of extraction and derivatization. The arginine, lysine, citrulline/threonine, and alanine peaks were taller in the sample processed with a single-step of extraction and derivatization, whereas the glutamine,

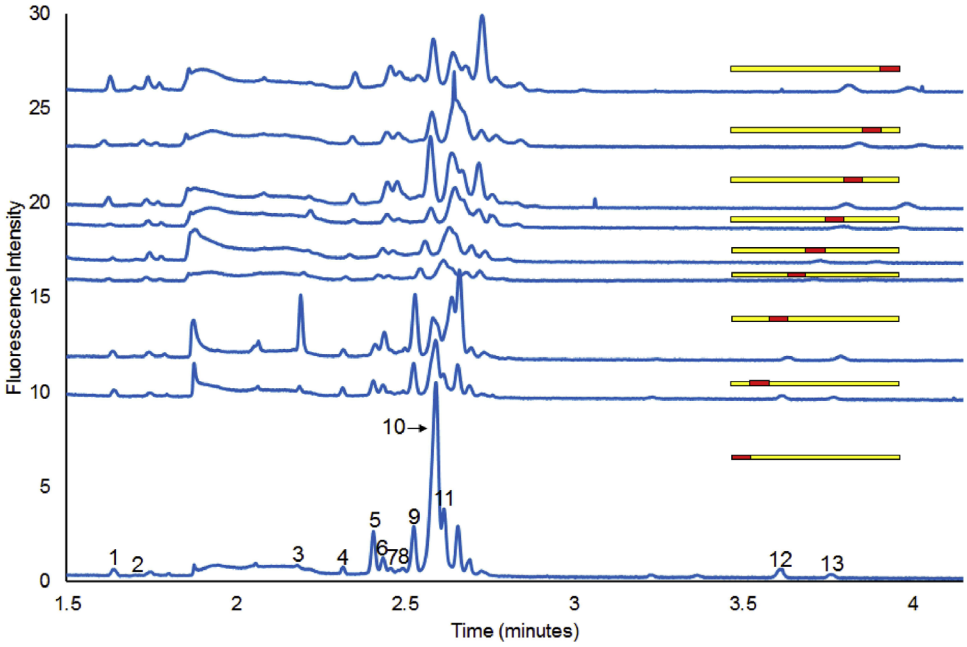

Figure 3. Electrophoretic separation of human tear primary amines absorbed by different regions of phenol red thread. After tear collection (0.8 µL), the wetted region of the thread was cut into nine pieces (2 mm), and each piece was vortexed and centrifuged for extraction in phosphate buffer (2 µL) before derivatization with CBQCA for fluorescence detection by capillary electrophoresis. The insets show the analyzed regions of the thread starting from the bottom with the tip inserted in the eye. The identified peaks and separation conditions are identical to those in Figure 2.

taurine, and glutamate peaks were taller after processing with extraction then derivatization.

Impact of Fiber Chromatographic Partitioning

To further investigate the impact of fiber thread intermolecular interactions on the analysis of tear primary amines, different regions of the tear-absorbed thread were analyzed separately by electrophoresis, and the percent normalized concentrations of 13 primary amine peaks were compared for individual samples collected on a thread ($n = 3$). As seen in the representative electropherograms in Figure 3, primary amines absorbed onto the thread partition in a chromatographic manner as tear fluid absorbs down the thread. Different regions of the thread capture different levels of tear primary amines. Figure 4 shows that arginine, histamine, citrulline/threonine, and serine had their highest concentrations in the last region of the thread, and significant changes were seen across thread regions in arginine, histamine, and citrulline/threonine. In contrast, glutamine, methionine, taurine, glycine, and glutamate had their highest concentrations in the first region of the thread, and significant changes were seen across thread regions in glutamine, taurine, glycine, and glutamate. Notably, positively charged lysine, polar asparagine, nonpolar alanine, and negatively charged aspartate all had similar proportions of their concentrations across the different regions of the thread.

Impact on Tear Small Molecule Quantitation

In this next experiment, the impact of thread processing on primary amine absolute concentration determination was investigated by assessing the quantitation of five standards when two different volumes are pipetted then eluted off the thread before derivatization and analysis compared with the same standards derivatized and analyzed directly in solution. The bar graph in Figure 5 shows that standards pipetted and eluted off the thread before derivatization and analysis have lower quantitation accuracy compared with standards derivatized and analyzed directly in-solution. Moreover, the error bars indicate that measurements of standards pipetted and eluted off the thread before derivatization and analysis have lower precision. This impact of on-thread processing leads to measured concentrations higher than actual concentrations for both pipetted volumes to varying degrees, where increasing the volume of standard

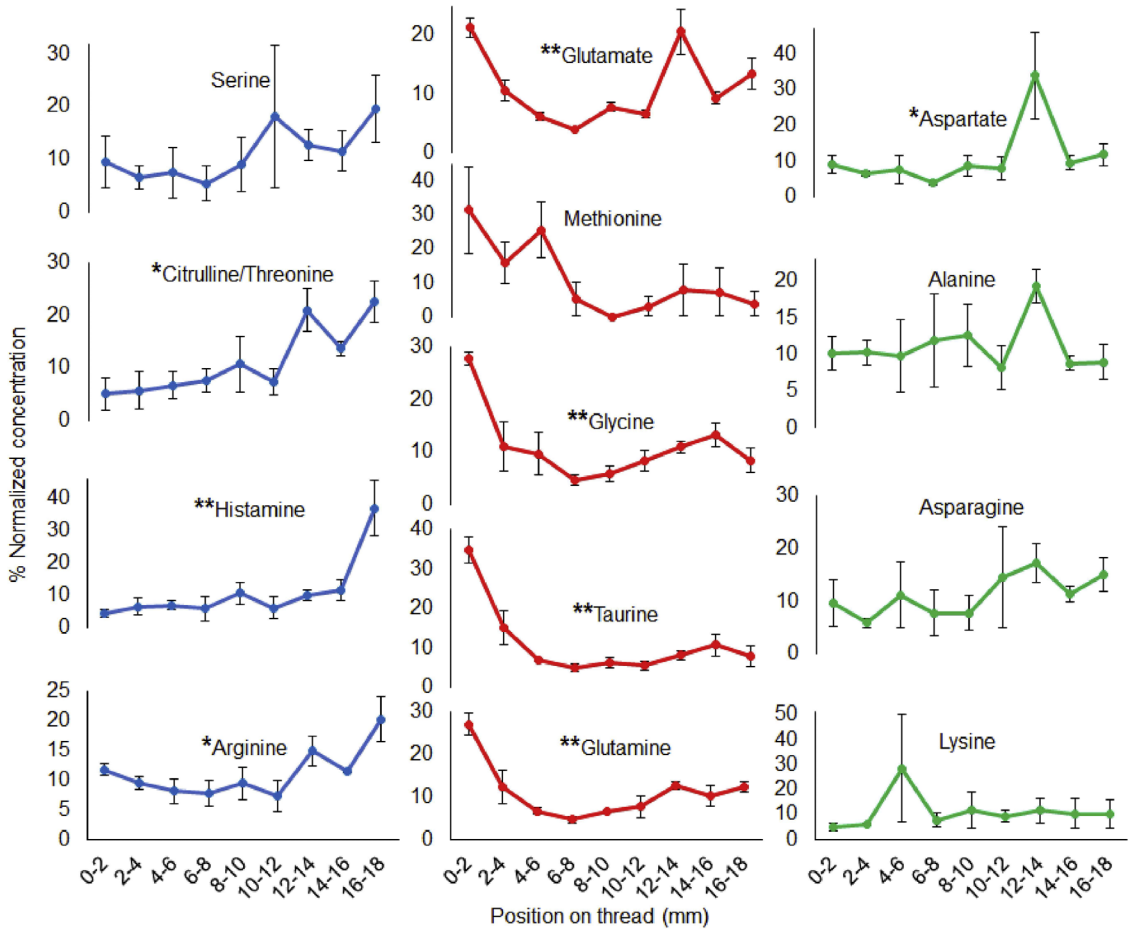

Figure 4. Comparison of 13 different primary amine-containing small molecule peaks across different regions of phenol red thread. Percent normalized concentrations represent percent peak height obtained from the ratio of the peak height in the electropherogram of interest to the sum of heights of the same peak across all electropherograms from individual thread-collected samples. Error bars represent standard errors of the mean ($n = 3$). One-way analysis of variance with repeated measures revealed significant changes across thread regions in arginine, histamine, citrulline/threonine, glutamine, taurine, glycine, glutamate, and aspartate (*$P < 0.05$ and **$P < 0.01$). Processing and separation conditions are those in Figure 3.

on-thread increased quantitation accuracy and precision for all five primary amines.

Impact of Collected Tear Volume

To evaluate the potential of improving thread-based tear quantitation by increasing sample volume, the impact of the collection of different tear volumes on small molecule electrophoretic separation resolution was investigated. In particular, two tear samples of different volumes collected identically and successively from the same subject were analyzed and compared. The representative electropherograms in Figure 6 show that the larger tear sample volume has significantly lower separation resolution and many unresolved peaks compared with the lower volume sample.

Discussion

Thread-based tear collection and processing significantly impact chemical analysis, leading to inconsistencies in tear profiles as well as quantitative inaccuracies depending on the protocol used. With the thread itself not producing a significant background signal, the differences seen between the representative electropherograms of tear samples collected by glass capillary tubes versus threads in Figure 2 suggest impacts of individual methods on chemical analysis. Between the thread-collected tear samples, the most likely reason for elution producing taller and sharper peaks, on average, compared with extraction is enhanced rinsing of tear primary amines from the thread by multistep elution, as we have previously reported.[15] The

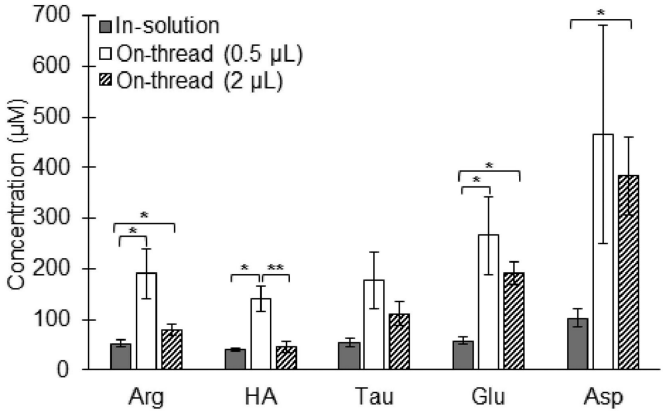

Figure 5. Comparison of five standard primary amine concentrations after direct in-solution derivatization and analysis or on-thread pipetting then elution before derivatization and analysis. In-solution refers to concentrations obtained from external calibration curves after directly derivatizing and analyzing the standards in-solution. On-thread refers to concentrations calculated by multiplying the obtained calibration curve concentration by the constant elution volume (4 × 2 µL = 8 µL) and dividing by the known pipetted volume that was completely absorbed by the thread (0.5 or 2 µL). All standards were prepared as 50 µM. All concentrations are averages, and error bars represent standard deviations ($n = 3$). The two-tailed unpaired Student *t*-test assuming unequal variances showed significant differences between measured concentrations of arginine, histamine, glutamate, and aspartate (*$P < 0.05$ and **$P < 0.01$). Arg = arginine; Asp = aspartate; Glu = glutamate; HA = histamine; Tau = taurine.

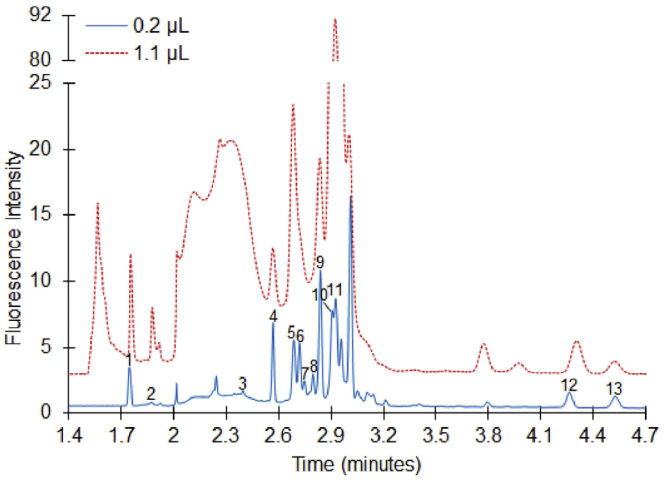

Figure 6. Comparison of primary amine separation resolution between human tear samples of two different volumes. Tear samples were collected successively and identically from the same subject via phenol red threads and processed with elution (4 × 2 µL phosphate buffer) then derivatized with CBQCA for fluorescence detection by capillary electrophoresis. Sample volume was determined from wetted thread length using an external calibration curve. The identified peaks, photomultiplier tube voltage, and separation conditions are identical to those in Figure 2.

differences between the two extraction methods may be caused by changes to derivatization or rinsing efficiency of some chemical components with direct addition of CBQCA to the wetted thread in the single-step of extraction and derivatization. Extraction is the most used processing method from Schirmer strip with different studies using different approaches. Previous studies have used elution to analyze proteins,[28] vortex mixing for only 15 minutes to analyze metabolites,[8] or multiple steps of extraction[9] or sonication[10] to analyze proteins. In addition, it was previously reported that less than 50% of absorbed protein was released from Schirmer strip after centrifugation at 10,000 rpm for 5 minutes, with the percent release varying significantly between different brands of the filter paper.[27] The results obtained here suggest that, for the given volume of rinsing buffer, processing with elution is superior to extraction for tear primary amine-containing small molecules analysis from thread. The observed rinsing differences between the identified primary amines may be caused by varying strengths of intermolecular interactions with thread cellulose, phenol red, phosphate rinse buffer, and other tear chemical components. Intermolecular interactions on Schirmer strip have also been reported to impact tear protein content extraction and analysis.[28] Together with these previous studies, this experiment shows that processing methods should be specially customized and optimized for specific tear analyte targets.

Interestingly, processing small pieces of the thread with extraction in Figure 3 leads to improved separation and allows resolution of asparagine, methionine, serine, and glycine, which were unresolved for both samples processed with extraction in Figure 2. This finding may be explained by the differences in extracted thread length and rinsing buffer volume between this (2 mm in 2 µL) and the previous (1.5 cm in 8 µL) experiment. These results further support the conclusion that processing methods should be customized for specific desired analyte measurements and applications. The preferential abundance of arginine, histamine, citrulline/threonine, and serine in the last region of the thread may indicate weak intermolecular interactions and cohesion to the thread, while that of glutamine, methionine, taurine, glycine, and glutamate in the first region of the thread may be indicative of strong intermolecular interactions and cohesion to the thread. The similar proportions of positively charged lysine, polar asparagine, nonpolar alanine, and negatively charged aspartate across the different regions of the thread may indicate complex intermediate cohesion to the thread. Another likely contributing factor to these observed trends is chromatographic overload. The occupation of thread

binding sites from molecules with strong cohesion to the thread may prevent molecules with weaker cohesion from binding and thus producing varying concentrations of different analytes across the thread. Because the change of solvent pH from the nearly neutral 7.1 of tears[31] to 8.0 of phosphate rinse buffer most likely alters the intermolecular interactions of different analytes significantly, these observations unlock several additional strategies for customized processing and selective analysis of potential target analytes. Moreover, the results of this experiment suggest that any single region of the wetted thread will not likely be representative of the whole sample for small molecule analysis. One previous study reported that the first three 5-mm segments of a Schirmer strip contained at least 80% of tear lipids regardless of wetted length, and the remaining wetted segments were discarded.[6] Another study has used a specially designed Schirmer strip with standardized punches of 4 mm in diameter that were cut out for protein and metabolite analysis, discarding the remaining wetted part of the Schirmer strip.[7] Although these approaches standardize collection volume, discarded tear-absorbed portions may include significant concentrations of some chemical components, and these discarded portions should be always assessed to evaluate the validity of the research protocol.

The overestimation of actual concentrations shown in Figure 5 is most likely owing to the evaporation of the phosphate rinse buffer during centrifugation and elution, which may explain the higher signal shown for some peaks of thread-collected samples compared with capillary tubes in Figure 2. This evaporation leads to a concentration increase observed in the capillary electrophoresis determination owing to the inaccurately larger elution volume used in back calculations. Increasing the volume of standard pipetted on-thread increases quantitation accuracy and precision most likely by reducing the error carried in back calculations. This increase in quantitation accuracy and precision with larger sample volumes may lay the framework for future improvements in the quantitation of thread-collected samples. Similar concentrating effect of proteins from Schirmer strip with low-volume samples after multistep centrifugation was reported previously.[29] The reason for the observed differences in quantitation accuracy between different primary amines is still unknown, but it may be related to thread intermolecular interactions as discussed in Figure 4. Specifically, the 2 µL on-thread samples of both arginine and histamine, which had weak cohesion to the thread, led to measured concentrations closer to the actual concentration compared with taurine and glutamate, which had strong cohesion to the thread. Both in-solution and on-thread processing led to relatively low accuracy and precision for aspartate. This likely reflects the poor fluorescence labeling of the amino acid and low sensitivity described previously by the low slope of aspartate calibration curve.[14,32] Importantly, the results of this experiment demonstrate that comparisons of absolute concentrations of tear components obtained using different absorption or processing methods may be unreliable without considering the impacts of each method on tear analysis.

The decrease in separation resolution with larger volume samples seen in Figure 6 may be explained in several ways. First, the lower separation resolution may be reflective of higher concentrations of primary amines in the larger volume sample. Decreasing the sample concentration by increasing the phosphate rinse buffer volume to obtain a higher separation resolution may be performed for large-volume samples. However, the collection of larger tear volumes introduces additional error in the determination of wetted thread length owing to absorbed tear evaporation during longer tear collection. Specifically, both tear absorption and evaporation from the wetted thread occur during tear collection. Although the rate of absorption is higher than evaporation, the longer collection times introduce an increasing extent of evaporative loss from the wetted thread. The impact from absorption leads to pH-based color changes across the thread, but the simultaneous tear evaporation slows the advancement of the absorptive front. This process results in measured wetted thread lengths that underestimate the total volume and chemical content collected. Absorbed tear evaporation was also previously reported to slow down the progression of the wetting front on Schirmer strip.[33] In addition, the collection of larger tear sample volumes is difficult with rodents and aqueous tear-deficient patients. Moreover, the larger volume sample likely had more unresolved tear proteins and other primary amine-containing large molecules as seen in Figure 6 at 1.5 minutes as well as between 2.0 and 2.5 minutes. Higher protein collection may be advantageous for tear protein analyses but likely decreases small molecule separation resolution. With the current separation conditions, the fluorescently labeled tear proteins appear as broad humps instead of sharp, characteristic peaks that potentially mask comigrating small molecule peaks. Furthermore, different primary amine-containing molecules can interfere and co-elute with each other, which complicates their quantitative analysis where separation conditions need to be methodically optimized to separate target molecules. More generally, regardless of the analysis method used, increasing tear

sample volume adds to the complexity of collection and analysis. These results outline the limitations and trade-off between obtaining high separation resolution and increasing quantitation accuracy by increasing sample volume with thread collection of tear samples.

In conclusion, this evaluation of the impacts of fiber thread-based tear sample collection and processing on primary amine analysis revealed significant alterations in the chemical composition of tear fluid. These results suggest that tear analysis methods can be customized for particular applications and analytes of interest. Furthermore, it is important to assess the chemical composition of any discarded portions of the absorbent material as well as process standards of known concentrations using the same methods to evaluate quantitative accuracy. With the growing interest in tear metabolite and protein profiling, these results identify potential inconsistencies and inaccuracies in the tear collection and analysis studies already published in the literature, as well as propose the standardization of different techniques in future studies.

Acknowledgments

Supported by the UIC Department of Chemistry, UIC President's Award Program STEM Initiative, Howard L. Kaufman Undergraduate Student Research Award (UIC LASURI), and UIC Honors College Undergraduate Research Grant.

Disclosure: **A. Barmada**, None; **S.A. Shippy**, None

References

1. Tiffany JM. Tears in health and disease. *Eye*. 2003;17:923–926.
2. Zhou L, Beuerman RW. Tear analysis in ocular surface diseases. *Prog Retin Eye Res*. 2012;31:527–550.
3. Barmada A, Shippy SA. Tear analysis as the next routine body fluid test. *Eye*. 2020, doi:10.1038/s41433-020-0930-0 [Epub ahead of print].
4. Nakatsukasa M, Sotozono C, Shimbo K, et al. Amino acid profiles in human tear fluids analyzed by high-performance liquid chromatography and electrospray ionization tandem mass spectrometry. *Am J Ophthalmol*. 2011;151:799–808.
5. von Thun Und Hohenstein-Blaul N, Funke S, Grus FH. Tears as a source of biomarkers for ocular and systemic diseases. *Exp Eye Res*. 2013;117:126–137.
6. Lam SM, Tong L, Duan X, Petznick A, Wenk MR, Shui G. Extensive characterization of human tear fluid collected using different techniques unravels the presence of novel lipid amphiphiles. *J Lipid Res*. 2014;55:289–298.
7. Dammeier S, Martus P, Klose F, et al. Combined targeted analysis of metabolites and proteins in tear fluid with regard to clinical applications. *Transl Vis Sci Technol*. 2018;7:22.
8. Chen L, Zhou L, Chan ECY, Neo J, Beuerman RW. Characterization of the human tear metabolome by LC-MS/MS. *J Proteome Res*. 2011;10:4876–4882.
9. Srinivasan S, Thangavelu M, Zhang L, Green KB, Nichols KK. iTRAQ quantitative proteomics in the analysis of tears in dry eye patients. *Investig Ophthalmol Vis Sci*. 2012;53:5052–5059.
10. Huang Z, Du CX, Pan XD. The use of in-strip digestion for fast proteomic analysis on tear fluid from dry eye patients. *PLoS One*. 2018;13:e0200702.
11. Zhou L, Zhao SZ, Koh SK, et al. In-depth analysis of the human tear proteome. *J Proteom*. 2012;75:3877–3885.
12. Lema I, Brea D, Rodríguez-González R, Díez-Feijoo E, Sobrino T. Proteomic analysis of the tear film in patients with keratoconus. *Mol Vis*. 2010;16:2055–2061.
13. Saijyothi AV, Angayarkanni N, Syama C, et al. Two dimensional electrophoretic analysis of human tears: collection method in dry eye syndrome. *Electrophoresis*. 2010;31:3420–3427.
14. Barmada A, Shippy SA. Thread-based assay for quantitative small molecule analysis of mice tear fluid by capillary electrophoresis. *Anal Bioanal Chem*. 2019;411:329–338.
15. Avilov V, Zeng Q, Shippy SA. Threads for tear film collection and support in quantitative amino acid analysis. *Anal Bioanal Chem*. 2016;408:5309–5317.
16. Nakamachi T, Ohtaki H, Seki T, et al. PACAP suppresses dry eye signs by stimulating tear secretion. *Nat Commun*. 2016;7:12034.
17. Wieser B, Tichy A, Nell B. Correlation between corneal sensitivity and quantity of reflex tearing in cows, horses, goats, sheep, dogs, cats, rabbits, and guinea pigs. *Vet Ophthalmol*. 2013;16:251–262.
18. Rentka A, Koroskenyi K, Harsfalvi J, et al. Evaluation of commonly used tear sampling methods and their relevance in subsequent biochemical analysis. *Ann Clin Biochem*. 2017;54:521–529.
19. Posa A, Bräuer L, Schicht M, Garreis F, Beileke S, Paulsen F. Schirmer strip vs. capillary tube

method: non-invasive methods of obtaining proteins from tear fluid. *Ann Anat*. 2013;195:137–142.
20. Shigeyasu C, Hirano S, Akune Y, Mochizuki H, Yamada M. Evaluation of the frequency of ophthalmic solution application: washout effects of topical saline application on tear components. *Curr Eye Res*. 2013;38:722–728.
21. Rohit A, Stapleton F, Brown SHJ, Mitchell TW, Willcox MDP. Comparison of tear lipid profile among basal, reflex, and flush tear samples. *Optom Vis Sci*. 2014;91:1391–1395.
22. Bitton E, Wittich W. Influence of eye position on the Schirmer tear test. *Contact Lens Anterior Eye*. 2014;37:257–261.
23. Calderone L, Grimes P, Shalev M. Acute reversible cataract induced by xylazine and by ketamine-xylazine anesthesia in rats and mice. *Exp Eye Res*. 1986;42:331–337.
24. Boehm N, Riechardt AI, Wiegand M, Pfeiffer N, Grus FH. Proinflammatory cytokine profiling of tears from dry eye patients by means of antibody microarrays. *Investig Ophthalmol Vis Sci*. 2011;52:7725–7730.
25. Choy CKM, Cho P, Chung WY, Benzie IFF. Water-soluble antioxidants in human tears: effect of the collection method. *Investig Ophthalmol Vis Sci*. 2001;42:3130–3134.
26. Li K, Chen Z, Duan F, Liang J, Wu K. Quantification of tear proteins by SDS-PAGE with an internal standard protein: a new method with special reference to small volume tears. *Graefes Arch Clin Exp Ophthalmol*. 2010;248:853–862.
27. García-Porta N, Mann A, Sáez-Martínez V, Franklin V, Wolffsohn JS, Tighe B. The potential influence of Schirmer strip variables on dry eye disease characterisation, and on tear collection and analysis. *Contact Lens Anterior Eye*. 2018;41:47–53.
28. Denisin AK, Karns K, Herr AE. Post-collection processing of Schirmer strip-collected human tear fluid impacts protein content. *Analyst*. 2012;137:5088–5096.
29. Sebbag L, McDowell EM, Hepner PM, Mochel JP. Effect of tear collection on lacrimal total protein content in dogs and cats: a comparison between Schirmer strips and ophthalmic sponges. *BMC Vet Res*. 2018;14:61.
30. Cabay MR, Harris JC, Shippy SA. Impact of sampling and cellular separation on amino acid determinations in *Drosophila* hemolymph. *Anal Chem*. 2018;90:4495–4500.
31. Coles WH, Jaros PA. Dynamics of ocular surface pH. *B J Ophthalmol*. 1984;68:549–552.
32. Patterson EE, Piyankarage SC, Myasein KT, et al. A high-efficiency sample introduction system for capillary electrophoresis analysis of amino acids from dynamic samples and static dialyzed human vitreous samples. *Anal Bioanal Chem*. 2008;392:409–416.
33. Telles R, Li W, Dursch TJ, Lin MC, Radke CJ. Human tear-production rate from closed-eye Schirmer-strip capillary dynamics. *Colloids Surf A Physicochem Eng Asp*. 2017;521:61–68.