Published in final edited form as:

Optom Vis Sci. 2010 November ; 87(11): 854–860. doi:10.1097/OPX.0b013e3181f6fb71.

Evaluation of Extractants and Precipitants in Tear Film Proteomic Analyses

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Introduction

Numerous proteins have been identified in the human tear film,¹ and the development of recent mass spectrometry-based identification methods have been instrumental in facilitating the description of the tear film proteome.^{2, 3} A lack of consensus in the scientific community remains regarding the functions of individual tear film proteins. Some of these functions may be beneficial in the protection of the ocular surface by their antimicrobial and inflammatory-related properties. The up- and down-regulation of tear film proteins may play a significant role in disease states like dry eye.⁴ In contact lens wear, it has been reported that significant levels of protein deposition has been identified as a potential cause of giant papillary conjunctivitis^{5–6} and also may be one of several contributory factors in the increased incidence of contact lens discomfort and intolerance.⁷

Lysozyme, first isolated in the human tear film in the 1920's,⁸ is commonly researched in the study of both tear film proteomics and contact lens protein deposition.^{9–15} Its abundance, small size (14.5 kDa) and positive charge allow these proteins to fit tightly into the structural matrix of negatively-charged FDA group IV contact lenses,^{16–18} making them difficult to extract. Research has shown significantly less deposition of proteins on silicone hydrogel lenses than on conventional lenses,^{10, 19} although more of the protein appears to be denatured than with conventional materials. Despite the reduction of biological deposits on these lenses, reports of contact lens intolerance due to protein deposition are still reported.⁵ Due to the small amount and large variation of individual proteins in the tear film and those deposited on hydrogel polymers, thorough and contemporary analyses of these tear proteins are providing more insight into their diversity.

Chemical solutions have been used to maximize the yields of available tear proteins for these further analyses.³ Chemical precipitants have been beneficial in pelletizing tear proteins so non-proteinaceous substances that may interfere with protein quantification assays (lipids or salts, for example) can be removed with the supernatant. In addition, chemical extractants have shown their usefulness in the removal of tear film proteins from

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Commercial Relationship Disclosures: The authors have no personal commercial interests in any of the aforementioned companies or products discussed in this work.

contact lenses^{11, 13} by breaking the absorbed proteins from the contact lens surface, yet, no study to date has compared the efficiencies of the various chemical treatments on the removal of proteins from both contact lenses and microcapillary-collected tears. The purpose of our study is two-fold. First, to compare the efficiency of protein extraction from a selected silicone hydrogel contact lens material using one of four chemical treatments evaluated in this study (acetone, trichloroacetic acid, urea, and trifluoroacetic acid/ acetonitrile). Second, to determine whether significant differences exist between the yields of chemically extracted or precipitated tear film proteins collected directly from the tears compared to samples not subjected to an extraction or precipitation procedure.

Materials and Methods

Subjects and Patient Sample

The study was approved by The Ohio State University Biomedical Institutional Review Board in accordance with the tenets of the Declaration of Helsinki. Informed consent and HIPAA documents were signed after explanation of all procedures. All subjects had previously been successful soft contact lens wearers with normal ocular health and a spherical contact lens prescription between +6.00 and -10.00 diopters. Information on demographics, medications, and standard medical questions were asked to determine inclusion criteria. Exclusion criteria included subjects less than 18 years of age, participation in investigational or device studies within seven days of enrollment, pregnancy, dry eye by the Contact Lens and Dry Eye Questionnaire (CLDEQ), and unacceptable fit or vision with study lenses.

Examination Sequence

Visit 1 (Day One)—Visual acuity with habitual spectacles was obtained from each eye of all study subjects. A subjective refraction was used to determine the contact lens power for contact lens fitting. Non-reflex tear samples of up to 5 μ L were collected from the inferior tear prism in each eye using either 2 or 5 μ L Drummond glass microcapillary tubes. The examiner wore powder free, non-latex gloves and used 16 $\times$ slit-lamp magnification. Ocular health assessment both with and without fluorescein staining was then conducted. Study lenses (Iotaafilcon B, O₂Optix, CIBA Vision, Atlanta, GA) were then fitted to the eyes and assessed for vision and fit. OptiFree RepleniSH (Alcon Labs, Fort Worth, TX) care solution was then dispensed to the subject with directions to use only this care system during the study and to use the system per manufacturer's guidelines (no rub, rinse five seconds each side). At the completion of the visit, subjects were instructed to wear the lenses for a minimum of 12 hours each day until the second visit 14 days later.

Visit 2 (Day 15)—Questions were asked to determine whether each subject followed study protocol, along with an updated medical history. Visual acuity, contact lens fit assessment, collection of contact lenses using powder-free non-latex gloves and sterile metal tweezers, and a slit lamp examination with fluorescein staining were then conducted.

Sample Preparation and Storage

The length of the tear collected inside each microcapillary was measured to calculate the approximate tear volume collected given the diameter of the microcapillary tube. Microcapillary-collected tear samples were then ejected into 1.5 mL clear plastic microcentrifuge tubes containing 20 μ L prepared tear storage buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA). The microcapillary was rinsed by pipetting the tear buffer and sample and then ejecting back into the microcentrifuge tube three times. Contact lenses collected at the second visit were stored dry in 4 mL sealed clear glass vials (Fisher Scientific, Dubuque, IA). All tear and contact lens samples were stored at -86°C until analysis.

Extraction and Precipitation Procedures

Chemical Extraction or Precipitation Solution Allocation—Based on the completion of the first visit, tear and contact lens samples for each subject were assigned to one of the four chemical solution groups. Microcapillary-collected tear samples from each eye within a subject were randomly assigned (Excel 2007, Microsoft Corp, Redmond, WA) across four groups of 10 subjects each to receive either one of the four chemical solutions or no extraction. One contact lens sample from these same 10 subjects was also assigned to chemical extraction using the same randomly allocated treatment used for the tears; thus, all four treatments were used with one contact lens from each subject. Samples containing tear storage buffer or unworn contact lenses only were subjected to one of the four solvents to evaluate for cross-reactivity (along with each of the precipitants/extractants alone) which may affect the tear protein quantification results.

Acetone Precipitation Procedure²⁰—Acetone was prechilled to -20°C and 100 μ L was then added to microcapillary-collected tear samples while 300 μ L was added to the contact lens samples. All samples were then vortexed and chilled for one (microcapillary-collected tear samples) or two (contact lens samples) hours at -20°C followed by 10–15 minutes centrifugation at 13,000 $\times g$, 4°C (accuSpin Micro R17, Fisher Scientific, Dubuque, IA). Pellets from tear samples and contact lens samples were washed with (100 μ L and 300 μ L, respectively) prechilled acetone (-20°C). Samples were then centrifuged a second time at the same speed and temperature for five minutes.

Trichloroacetic Acid (TCA) Precipitation Procedure²¹—TCA (20% concentration) was added to microcapillary-collected tear samples in 100 μ L amounts while 300 μ L was added to the contact lens samples. All samples were allowed to cool in an ice bath for 30 minutes. The supernatant was evaporated from the samples by vacuum centrifugation (VacufugeTM Concentrator 5301, Eppendorf, Westbury, NY). Prechilled acetone (-20°C) was added to all samples in the amounts previously described in the acetone procedure. Vacuum centrifugation evaporated the supernatant from the samples.

Urea Extraction Procedure¹³—Fresh extraction buffer was prepared (80% urea, 0.1 M Tris HCl pH 8.0, 2 mM EDTA, 1 mM dithiothreitol) and 100 μ L was added to microcapillary-collected tear samples while 500 μ L was added to the contact lens samples. Tear samples were incubated with shaking at 95°C , 300 rpm (Thermomixer R, Eppendorf,

Westbury, NY) for 30 minutes while contact lens samples were incubated at the same temperature and speed for 4 hours. All samples were then allowed to cool in an ice bath for 30 minutes, and then the supernatant was evaporated off with vacuum centrifugation.

Trifluoroacetic Acid/Acetonitrile (TFA/ACN) Extraction Procedure¹¹—A 50:50 mix of 0.2% TFA/ACN was prepared and 100 μ L were added to tear samples and 1 mL was added to contact lens samples. All samples were then chilled at -20°C for 16 hours. The supernatant for all samples were evaporated off with vacuum centrifugation.

Post-Chemical Treatment Preparation—Following aspiration of the supernatant by aspiration (acetone) or evaporation (TCA, urea and TFA/ACN), the pellets were resuspended with 25 μ L High Performance Liquid Chromatography (HPLC)-grade water (except for 25 μ L urea buffer for urea extracted samples) and reserved for protein quantification described below.

Second Contact Lens Extraction

Contact lenses, except those subjected to acetone, were reserved following the first extraction and stored in their original sealed glass vials at -86°C until a second extraction procedure was performed. The extraction procedures for each solution were performed the same as for the first extraction in order to help determine the efficacy of a single chemical extraction.

Protein Quantification

A Bradford assay²² was conducted to determine the quantity of tear film proteins in tear, contact lens samples, and controls. Bradford reagent (Sigma-Aldrich, St. Louis, MO) was added to three 5 μ L aliquots of each sample as well as to wells each containing exclusively HPLC-grade water, urea or TFA/ACN buffer alone in a 96-round well plastic assay plate. The total protein concentration of all tear and contact lens samples was determined by measuring the peak absorbance at 595 nm (Infinite M200, Tecan, Durham, NC) in triplicate. The protein concentrations of the three aliquots for each sample were then averaged. Protein quantities were determined by extrapolating the mean protein concentration data from the standard curve. (The standard curve, with Bovine Serum Albumin (Thermo Scientific, Rockford, IL) used as the standard, was constructed by subtracting the concentration of the blank from several pre-determined concentrations.) All protein quantities were recorded in $\mu\text{g}/\mu\text{L}$ for tear samples and $\mu\text{g}/\text{lens}$ for contact lens samples.

Statistical Analyses

Given that there were 10 subjects assigned to each of the four chemical treatment groups, nonparametric statistical tests (Wilcoxon Signed Rank, Kruskal-Wallis one-way ANOVA, and Mann-Whitney) were performed by computer using Small Stata 10.0 (Stata Inc., College Station, TX). Descriptive statistics including means and standard deviations were used to characterize the data. Comparisons of protein concentrations were made between untreated and chemically-treated microcapillary-collected tear samples, between treatments across contact lens sample groups and, for contact lens samples, to compare the first and

second extractions for each of the four solutions used in this study. Spearman Rank Correlation was performed to evaluate the relation between tear protein concentration and microcapillary-collected tear volume for samples exposed and non-exposed to a treatment as well as for all samples combined.

Results

Subject demographics

Forty subjects were enrolled in the study, with all subjects completing both first and second visit (100%). The average age was 28.1 ± 7.1 years (median, 25.5 years; range, 20–57 years) and 34 of the subjects were female (85%).

Microcapillary-Collected Tear Samples

Table 1 shows the average protein concentrations from microcapillary-collected tear samples receiving either chemical treatment or no treatment. There were generally statistically significant reductions in tear protein concentration from samples subjected to a chemical extraction or precipitation step (all $p < 0.007$). Negligible cross-reactivity occurred between the tear storage buffer only and the precipitant acetone and the urea extractant ($0.01 \mu\text{g}/\mu\text{L}$ and $0.03 \mu\text{g}/\mu\text{L}$, respectively) as well as with the urea extractant only ($0.02 \mu\text{g}/\mu\text{L}$). Tear samples subjected to acetone precipitation had the highest amount of protein recovered out of the four chemical methods evaluated when compared to untreated tear samples. Out of the four extraction or precipitation solutions tested, samples assigned to the acetone precipitation had the most recoverable available protein when compared to the TCA, urea, and TFA/ACN.

Tear Volume and Protein Concentration of Microcapillary-Collected Tear Samples

For microcapillary-collected tear film samples that did not go on to receive precipitation or extraction, the approximate average volume of tear fluid collected was $3.9 \pm 1.3 \mu\text{L}$, while samples that went on to be treated using one of the four solutions was $3.7 \pm 1.5 \mu\text{L}$. When comparing tear volume of microcapillary-collected tear samples and the protein concentration from those samples, there was a significant positive relationship between protein concentration and tear volume collected for all samples combined ($r = 0.39$, $p = 0.0003$). The same positive relationship was true for tear protein concentrations compared to total tear volume collected from both untreated ($r = 0.48$, $p = 0.002$) and treated ($r = 0.42$, $p = 0.006$) samples.

Contact Lens Samples-Initial Extraction

The average amounts of tear protein extracted from the lotrafilcon B material by the four chemical treatments can be seen in Figure 1. Except for the acetone vs. urea treatment groups ($p = 0.29$), there was a significant difference in the amount of protein removed from contact lenses compared to the other three solutions (all $p < 0.005$) employed in this study. TFA/ACN ($8.04 \pm 3.75 \mu\text{g}/\text{lens}$) yielded an average of 2.4, 3.9, and 27.0 times the amount of available protein from the lenses compared to those subjected to urea ($3.29 \pm 2.32 \mu\text{g}/\text{lens}$), acetone ($2.06 \pm 1.15 \mu\text{g}/\text{lens}$), and TCA ($0.31 \pm 0.23 \mu\text{g}/\text{lens}$), respectively. Out of the four solvents evaluated, only the unworn contact lens subjected to urea extraction revealed cross-

reactivity, although it only attributed to 6% of the mean tear protein recovered from worn contact lenses (0.20 µg/lens).

Contact Lens Samples-Secondary Extraction

The average tear protein yielded from a second removal step using the same solutions (TCA, urea, and TFA/ACN) can also be seen in Figure 1. A second acetone procedure could not be conducted as the lenses did not remain intact following the initial treatment. Only one of the ten samples to receive the second TCA procedure was found to have a measurable protein concentration (2.51 µg); however, it is believed this sample became contaminated for an undetermined reason during the Bradford assay, and the result was excluded from statistical analysis. Unlike the first extraction, average protein concentration from urea-extracted (1.94 ± 0.31 µg/lens) contact lens samples was greater than those extracted with TFA/ACN (1.64 ± 0.33 µg/lens), and the differences were statistically significant ($p = 0.02$).

Discussion

Microcapillary-Collected Tears Comparisons

This study may be one of the first to report on comparisons of tear protein yields from samples subjected to an extraction or precipitation solution. The small sample size of non-reflex tears has been identified as one of the limiting factors in the analysis of the human tear proteome.² The minute quantity of tear protein recovered from individual tear and contact lens samples often poses a challenge in performing further proteomic analyses like gel electrophoresis. To address this issue, individual samples are often pooled in order to increase volume or concentration of tear proteins. A point of concern with pooled samples is the probability of bias in the interpretation of a test result as pooling samples obscures biological variability that may otherwise be important. Additionally, with pooled samples, individual clinical test results can no longer be correlated to biological findings. Identifying methods of optimizing tear protein yields for these further analyses can help alleviate the need to pool samples, allowing for the evaluation of tear proteins on an individual basis.

Tear protein quantification has primarily been conducted on samples not previously subjected to a chemical precipitation solution.^{23–26} In this study, over four times more tear protein was recovered from tear samples compared to samples subjected to an extraction or precipitation step. Two plausible explanations for the significant reduction in tear proteins when using precipitating solutions may be traced to the solution itself. First, acetone, which provided the highest mean yield of recoverable tear proteins out of the four methods evaluated, precipitated enough proteins to form a visually identifiable pellet. Despite the care taken to avoid disturbing the pellet, some protein may have been lost during removal of the supernatant. With the other three solutions evaluated in this study (TCA, urea, and TFA/ACN), the poor yields may be attributed to the simple inability of these solutions to remove or precipitate proteins from the tear solution; all of these samples had minimally-visible protein pellets following evaporation.

Isolated reports^{3, 27} of the use of chemical precipitation solutions on microcapillary-collected tear samples have previously been reported in the scientific literature with mixed

results. A recent study by Green-Church and coworkers³ recovered nearly 7 $\mu\text{g}/\mu\text{L}$ of available tear protein from pooled microcapillary-collected tear samples by acetone, over twice the amount found in this study. The collection of reflex tears or the assumption that elevated tear protein levels are seen in pooled tear samples compared to non-pooled samples of the same volume may be two possibilities that account for these differences in yields. In another study using TCA, Meijer and van Haeringen²⁷ reported poor tear protein yields by a modified turbidimetric assay from tear samples; the authors implied that a soluble glycoprotein found in the human tear film may actually inhibit TCA from precipitating tear proteins. Our data revealed poor yields with TCA as well, even with the Bradford protein quantification assay used in this study. It has been reported in the literature that the inclusion of a sonication step using a buffer containing urea, thiourea, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and dithiothreitol (DTT) following TCA precipitation increases protein recovery from hippocampal neurons,²⁸ although this has not yet been demonstrated in the laboratory with tear proteins. Another study by Jiang and colleagues reported that TCA precipitation and subsequent ultrafiltration provided a more efficient sample concentration from human plasma samples for two-dimensional (2D) gel electrophoresis compared to chloroform/methanol, which was not evaluated in this study.²⁹ Both of these findings may suggest that TCA is a more suitable for use in samples known to contain high levels of proteins, or for the removal of proteins not normally found in the human tear film. To the authors' knowledge, no previous reports in the literature have provided a perspective on the use of either urea or TFA/ACN in the extraction of proteins from human tears.

Tear Protein Concentration vs. Volume

It was expected that increased tear protein concentration would have been seen with increased collected tear volume; however, there was uncertainty if this assumption held true for samples receiving a chemical extraction or precipitation step as well (although there was nearly an equal average tear volume collected from both groups). Despite the potential for protein loss or denaturation by the chemical solution, the positive relationship between protein concentrations and tear volume for both treatment groups were similar and statistically significant for both tear samples exposed or non-exposed to one of four chemical solutions evaluated.

Extracted Tear Proteins from Contact Lens Comparisons

Previous studies have shown a significant reduction in the quantity of tear protein extracted from silicone hydrogel lenses compared to conventional (non-silicone) hydrogels.^{10, 19} In addition, Subbaraman and colleagues noted a decrease in the extraction efficiency of lysozyme among several silicone hydrogel lens materials (including lotrafilcon B) compared to the FDA Group IV material etafilcon A (Acuvue, Vistakon Inc, Jacksonville, FL).¹⁵ Several possibilities for the differences in concentrations extracted from these lenses may be explained by several properties including intrinsic positive surface charge, small pore size, low water content, the presence of siloxane groups, and surface treatments of silicone hydrogel lenses.³⁰

Numerous studies evaluating chemical extractants on conventional hydrogels^{11, 13, 14, 19, 31–36} and, most recently, silicone hydrogel lenses,^{9, 10, 14, 15, 19, 30, 37–39} have been reported in the scientific literature. The majority of the research has primarily focused on two used in this study: urea buffer¹³ and TFA/ACN.¹¹ The protein denaturant sodium dodecylsulfate (SDS), which was not used in this study, has reportedly been very efficacious in removing proteins from conventional hydrogel lenses, but ineffective in extracting tear proteins from silicone hydrogel lenses.¹⁹

TFA/ACN extraction significantly increased the quantity of recoverable protein from lotrafilcon B contact lenses compared to the other chemical extraction methods tested. It has been reported the amount of tear protein extracted *in vitro* from lotrafilcon silicone hydrogel contact lenses by TFA/ACN was <10 µg/lens after approximately 14 days of incubation.^{30, 39} These quantities are similar to the results from our *in vivo* study even though *in vitro* studies rely on the use of artificial tear solutions that may artificially increase protein recovery. More studies need to be conducted to investigate protein deposition on silicone hydrogels *in vivo*.

Using urea as an extraction buffer, Zhao et al.⁴⁰ reported recovering an average of 1.7 ± 2.3 µg/lens from lotrafilcon B lenses after 30 days of daily wear (at least 5 days/week, 6 hours/day) with the OptiFree Replenish care regimen (rinse, no-rub) by bicinchoninic (BCA) assay. These values are fairly similar to the findings from our study despite the shorter wear time in our study and use of an alternative protein quantification assay. A longer wearing period may reduce the amount of recoverable proteins by chemical extraction due to the proteins becoming further imbedded into the structural matrix of the contact lens. Despite the high level of sensitivity and accuracy of Bradford and BCA assays, the level of agreement between these two assays is uncertain, although it has been reported by Ng and colleagues that reports of tear protein quantification have varied based on assay (Bradford, Lowry) or standard used (BSA, human albumin serum, bovine immunoglobulin G).⁴¹

Nearly 10 µg/lens was extracted with acetone from lotrafilcon B silicone hydrogel lenses following 14 days of daily wear using the AQUify (CIBA Vision, Inc) care solution (no rub, rinse only) compared to our study with the RepleniSH care system.³⁷ A lower yield of recoverable protein may be attributable to the differences in solution efficacy.

As stated previously, all extraction and precipitation methods (including the volumes of the individual solutions) employed in this study have been reported in the literature.^{11, 13, 20, 21} Varying the volume can affect the amount of protein extracted or precipitated from the tear solution. One reason for the increased amount of recovered tear protein from TFA/ACN-treated contact lens samples over those samples subjected to urea, TCA, or acetone may be attributed to the volume of solvent used (1 mL TFA/ACN compared to 500 µL for urea and 300 µL for TCA and acetone). Increasing the volume of the solution used may affect the efficiency of the extraction (or precipitation) by increasing the amount of recoverable protein.

Secondary Contact Lens Extraction

Little is known about the impact of a second extraction on the yield of tear protein when using the same chemical solution and protocol as was used in the initial extraction. Far less protein was recovered with both urea and TFA/ACN, however, the findings were highly significant with TFA/ACN and only marginally so with urea. These findings suggest that TFA/ACN is more efficient in extraction (i.e., the first extraction yielded far more than the second extraction) of tear proteins from lotrafilcon B than urea (i.e., both the first and second extractions yielded relatively low levels, with the second yielding slightly higher than the first). A second extraction step with TFA/ACN may be beneficial in recovering additional tear proteins, particularly tear proteins present in minute quantities, for use in further proteomic analyses.

In summary, this study is one of the first to report on both the comparisons of extraction and precipitation solutions used in tear film and contact lens proteomic studies. Untreated tear samples provided the most available tear protein compared to those samples subjected to any one of the chemical extraction or precipitation treatments evaluated in this study. Treated, untreated, and combined tear samples had significantly increased tear protein concentration compared to the increasing amount of tear volume collected. TFA/ACN chemical extraction from lotrafilcon B silicone hydrogel contact lenses offered the highest yield of available tear protein (nearly 8 µg/lens average) out of the four solutions evaluated. A secondary extraction procedure with urea or TFA/ACN may be beneficial in removing additional tear proteins from this type of silicone hydrogel lens. Although urea has the tendency to adversely affect the results of the Bradford assay following protein extraction from a contact lens, the error appears to be minimal. There appears to be no substantial evidence that the remaining solvents adversely affect protein quantification results from the Bradford assay. In light of these results, caution should be observed when a particular protein of interest is to be studied. Functional studies like gel electrophoresis (1D) with densitometry or determination of the absolute amount of a particular protein both before and following extraction or precipitation, may provide more insight into the distribution of tear proteins in a sample depending on the extraction or precipitation method used. In other words, a higher yield of an individual tear protein of interest could possibly be obtained from either one of the alternative procedures that did not yield the most available tear protein (acetone, TCA, or urea) or by, for example, the addition of an acetone precipitation step following TFA/ACN extraction. (The acetone wash step is beneficial when gel electrophoresis studies are to be conducted following protein quantification as the sample is less likely to contain contaminants that may adversely affect the appearance of the individual protein bands). Further research is needed to identify novel chemical extraction and precipitation solutions or methods as a way of improving the efficiency of tear protein recovery from both tears and contact lens materials.

Acknowledgment

The authors would like to thank Kathleen Reuter, OD of The Ohio State College of Optometry for her assistance with data collection.

Grant Support: None

Dr. Jason Nichols has received research funding from Alcon Laboratories, Inc, CIBA Vision, Inc, Inspire Pharmaceuticals, OcuSense, and Vistakon, Inc within the last year. Dr. Kelly Nichols is a consultant to Inspire Pharmaceuticals, Allergan, and Pfizer and has received research funding from Alcon, Inspire Pharmaceuticals, OcuSense, and Pfizer in the last year. Dr. Chandler is a consultant to Vistakon, Inc.

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Figure 1.

Contact Lens Samples. Average protein concentration (µg/lens) estimates for extracted contact lens samples by assigned treatment group following the first ($\chi^2 = 26.61$, $p < 0.001$) and second extraction ($\chi^2 = 5.67$, $p = 0.02$).

* Significantly different from TFA/ACN following the first treatment ($p < 0.008$). And, with the exception of acetone vs. urea ($p = 0.29$), the average protein concentration between the other comparisons (acetone vs. TCA and urea vs. TCA) were significantly different ($p < 0.001$).

† A second treatment was not conducted on acetone or TCA samples.

‡ Significant difference in protein yields within each treatment between the first and second extraction were observed for both urea ($p = 0.04$) and TFA/ACN ($p = 0.005$).

Table 1

Microcapillary-collected tear protein samples. Average protein concentration estimates ($\mu\text{g}/\mu\text{L}$) for both treated (with a precipitant or extractant) and untreated tear protein samples.

Treatment	Treated Average $\pm$ SD	Untreated Average $\pm$ SD	Average % Difference	Wilcoxon Signed Ranks p-value
Acetone Precipitation	2.82 ± 2.02	5.70 ± 3.78	50.4	0.005*
TCA Precipitation	1.34 ± 0.83	5.67 ± 1.85	76.3	0.005*
Urea Extraction	0.57 ± 0.26	5.97 ± 3.70	90.5	0.007*
TFA/ACN Extraction	0.72 ± 0.18	5.38 ± 2.06	86.6	0.005*
Overall	1.36 ± 1.39	5.68 ± 2.88	76.0	<0.0001*

* Significantly different