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Targeted Workflow Investigating Variations in the Tear Proteome by Liquid Chromatography Tandem Mass Spectrometry

Maggy Lépine, Oriana Zambito, and Lekha Sleno*

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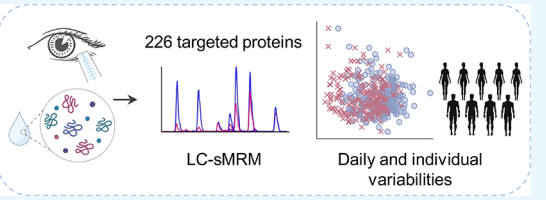
ABSTRACT: Proteins in tears have an important role in eye health and have been shown as a promising source of disease biomarkers. The goal of this study was to develop a robust, sensitive, and targeted method for profiling tear proteins to examine the variability within a group of healthy volunteers over three days. Inter-individual and inter-day variabilities were examined to contribute to understanding the normal variations in the tear proteome, as well as to establish which proteins may be better candidates as eventual biomarkers of specific diseases. Tear samples collected on Schirmer strips were subjected to bottom-up proteomics, and resulting peptides were analyzed using an optimized targeted method measuring 226 proteins by liquid chromatography-scheduled multiple reaction monitoring. This method was developed using an in-house database of identified proteins from tears compiled from high-resolution data-dependent liquid chromatography tandem mass spectrometry data. The measurement of unique peptide signals can help better understand the dynamics of each of these proteins in tears. Some interesting trends were seen in specific pathways or protein classes, including higher variabilities for those involved in glycolysis, glutathione metabolism, and cytoskeleton proteins and lower variation for those involving the degradation of the extracellular matrix. The overall aim of this study was to contribute to the field of tear proteomics with the development of a novel and targeted method that is highly amenable to the clinical laboratory using high flow LC and commonly used triple quadrupole mass spectrometry while ensuring that protein quantitation was reported based on unique peptides for each protein and robust peak areas with data normalization. These results report on variabilities on over 200 proteins that are robustly detected in tear samples from healthy volunteers with a simple sample preparation procedure.



INTRODUCTION

Tears represent a complex biofluid found at the surface of the eye, with an inner mucin layer, a middle aqueous layer, and an outer lipid layer.[1] Altogether, these three layers are composed of proteins, endogenous peptides, metabolites, electrolytes, glycoproteins, and lipids.[2,3] These various molecules play several important roles in the maintenance of eye health. The tear film protects the eye against infection[4] and ensures adequate lubrication and nutrition of the ocular surface.[5] Proteins in tears, mainly contained in the aqueous phase, play a central role where a change in the quantity and quality of proteins at the ocular surface has previously demonstrated correlation with ocular disease.[1,6−10]

Over the years, the tear proteome has become a promising source of biomarkers for various eye diseases, with mass spectrometry (MS) leading the way as a powerful tool for protein identification and quantitation. Tear samples can be collected non-invasively on Schirmer strips, a great advantage for clinical diagnosis and evaluation. This strategy has been applied previously to eye diseases[8] and even some neurodegenerative diseases.[11,12] This technique of sampling tears and profiling proteins with mass spectrometry can be useful in establishing which proteins can be robustly detected in a population without the presence of known disease. The current study was designed to identify tear proteins using a bottom-up proteomics approach and establish which proteins can be robustly detected in non-diseased volunteer samples and how they vary between individuals and within the same individual collected on different days.

Previous studies have been focused on the analysis of tear proteome using different mass spectrometry-based methods. To date, a few studies have focused on the identification of proteins by liquid chromatography tandem mass spectrometry (LC−MS/MS) in tear samples collected from individuals with no eye disorders. These studies examined pooled samples from different volunteers,[13−17] from a single volunteer,[4] or investigated specific differences in protein profiles based on collection methods.[16] Protein composition in tears has been shown to vary depending on gender, age, and sampling time, using two-dimensional electrophoresis[18] and chip-based arrays.[19] A previous untargeted nanoLC-HRMS/MS study found 17 proteins that correlated with increased age in 115

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patients aged between 18 and 83 years old.[20] A 2015 study by Tong *et al.* used nanoLC coupled to high-resolution tandem mass spectrometry to target 47 tear proteins, eight of which were quantified using isotope-labeled peptide standards in samples from 10 volunteers.[21] A recent study looked at the variabilities in two individuals sampled in the morning and afternoon on three different days with an untargeted nanoLC-Orbitrap high resolution MS/MS method and found that proteins involved in the immune system were found to be elevated in the morning samples.[22]

Mass spectrometry-based proteomics of complex biofluids is now a common practice in clinical biomarker studies,[23,24] where the change in specific protein levels in patients can help diagnose specific diseases or inform on treatment outcomes. In quantitative studies involving a list of targeted proteins, liquid chromatography coupled with multiple-reaction monitoring (MRM) detection using triple quadrupole platforms is considered as the gold standard.[25] More recently, with the advancement of high-resolution mass spectrometry techniques, data-independent acquisition has become popular for quantitative untargeted proteomics.

In this study, initial high-resolution tandem mass spectrometry analyses were used to compile a database confidently identified peptides from tear proteins. Following the development of LC-MRM methods to investigate which proteins were consistently well detected in 31 different samples, the inter-day and inter-individual variations of 226 tear proteins from nine volunteers with no known eye diseases were examined.

EXPERIMENTAL METHODS

Chemicals. Sequencing grade modified trypsin was obtained from Promega (product V5111, Madison, WI, USA). HPLC-grade acetonitrile (ACN) and methanol (MeOH), ammonium hydroxide (30%, NH_4OH), ammonium bicarbonate (ABC), ammonium acetate (NH_4OAc), formic acid (FA), iodoacetamide (IAM), and dithiothreitol (DTT) were all obtained from Sigma-Aldrich (Oakville, ON, Canada). Ultrapure water was from a Millipore Synergy UV system (Billerica, MA, USA).

Sample Collection. Tears were collected from volunteers without eye complaints or known disorders using Schirmer sterile tear strips (World Sports Vision), filling the strip to 23 mm. The first 3 mm was removed (to avoid contamination from skin proteins as well as other interferences from residues from cosmetics, for example), with the remaining 20 mm cut into four pieces and placed into a polypropylene tube (Eppendorf, Mississauga, ON, Canada) prior to storing at −80 °C. Samples from 12 volunteers (six women, six men, 37 ± 11 years old) were used to compile an in-house database of identified proteins. A subsequent set of samples from 16 subjects (6 women and 10 men, 34 ± 11 years old) was then used for targeted liquid chromatography-scheduled multiple reaction monitoring (LC-sMRM) analyses on 596 protein groups (split into two methods), from which 226 proteins were selected based on having robust, well-detected peptide signals in all samples. Inter-day and inter-individual variabilities of these 226 proteins were evaluated using one optimized LC-sMRM method from nine volunteers (five women, four men, 30 ± 6 years old), on three different days. All samples were collected in accordance with the UQAM Research Ethics Committee, following standards for research involving humans (ethics certificate CERPE-4790), including signed consent.

Sample Preparation. Samples were incubated in 200 μL of ABC buffer (100 mM, pH 8.5) for 15 min in an ultrasonic bath followed by the addition of 5 μL of 100 mM DTT (20 min at 37 °C) and 10 μL of 100 mM IAM (30 min at 37 °C in the dark) for reductive alkylation prior to digesting with 1 μg of trypsin for 16 h at 37 °C. Resulting peptides were diluted with water (to 1 mL) prior to solid-phase extraction (SPE) on OASIS HLB cartridges (30 mg/1 mL) from Waters Limited (Mississauga, ON, Canada). Cartridges were conditioned with 1 mL of MeOH followed by 1 mL of water. Samples were loaded and washed with 1 mL of water before elution with 2 additions of 500 μL of MeOH. Extracts were dried with a universal vacuum concentrator (Fisher Scientific, Mississauga, ON, Canada) and reconstituted in 110 μL of 10% ACN, 0.2% FA.

HPLC Peptide Fractionation. Left and right eye tear samples from one volunteer were digested and combined followed by SPE-HLB extraction (as above). The dried extract was then reconstituted with 120 μL of 10 mM NH_4OAc (pH 10), and 100 μL was injected onto a ZORBAX Extend-C18 column (5 μm, 250 × 4.6 mm, Agilent Technologies, Palo Alto, CA, USA) using an Agilent 1200 Series HPLC. High-pH fractionation was performed at 0.6 mL/min using a gradient of 10 mM ammonium acetate (pH 10, adjusted with NH_4OH) in water (A) and 10% A/90% ACN (B). The gradient started at 5% B held for 2 min, increased to 50% in 12 min, to 70% within 0.5 min, and was held at 70% for another 6.5 min. UV absorbance was monitored at 220 and 280 nm during fractionation. Sixteen fractions of 660 μL were collected, starting from 3 min after injection, and concatenated into eight fractions as follows: fraction 1 + 9, 2 + 10, 3 + 11, *etc.* Samples were then dried and reconstituted in 50 μL with a solution of 0.1% FA in 5% ACN.

MCX Peptide Fractionation. For samples subjected to SPE-MCX fractionation, digests from right and left eyes were combined and diluted to 1 mL with 2% FA prior to SPE on Waters OASIS MCX cartridges (30 mg). Cartridges were conditioned with 1 mL of MeOH and 1 mL of water. Acidified samples were loaded and washed with 1 mL of 2% FA followed by 1 mL of MeOH and 1 mL of 10 mM NH_4OAc in 50% MeOH. Samples were eluted in five different fractions using 1 mL of 50% MeOH each containing 20 mM NH_4OAc (fraction 1), 25 mM NH_4OAc (fraction 2), 50 mM NH_4OAc (fraction 3), 0.1% NH_4OH (fraction 4), and 3% NH_4OH (fraction 5). The five fractions were dried and reconstituted in 110 μL in 10% ACN, 0.2% FA.

LC−High-Resolution MS/MS Analyses. Samples were injected (25 μL) onto an Aeris PEPTIDE XB-C18 (1.7 μm, 100 × 2.1 mm) with a SecurityGuard ULTRA C18-peptide guard (Phenomenex, Torrance, CA, USA) using a Nexera UHPLC system (Shimadzu, Columbia, MD, USA). Gradient elution was performed at 40 °C at 0.3 mL/min using a gradient of water (A) and ACN (B), both containing 0.1% FA. Initial conditions were set at 5% B for 2.5 min, increased to 30% over 37.5 min, up to 50% in 2 min, 90% within 2 min, and held for 3 min before re-equilibration of the column (8 min). A TripleTOF 5600$^+$ (quadrupole-time-of-flight) mass spectrometer (Sciex, Concord, ON, Canada) equipped with a DuoSpray ion source in positive electrospray mode was used for high-resolution mass spectrometry (HRMS) analysis. Both information-dependent acquisition (IDA) and data-independent acquisition (SWATH) were performed. The ion source parameters were set at 35 psi for curtain gas, 50 psi for

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nebulizer (nitrogen) and drying (dry air) gases (GS1 and GS2), 500 °C for source temperature, 5000 V for ion spray voltage, and 80 V for declustering potential (DP). Automatic calibration was performed with a calibrant delivery system every four injections using an in-house standard mix, for TOF-MS and high sensitivity MS/MS modes (2 min method), with infusion through the APCI probe of the source, with make-up flow from the ESI probe at initial gradient conditions.

For IDA experiments, TOF-MS data was first acquired from m/z 140 to 1250 with an accumulation time of 250 ms, followed by MS/MS acquisition from m/z 80 to 1300, of the 15 most intense ions between m/z 300 and 1250 with exclusion of 20 s after two occurrences, a threshold of 250 cps, and dynamic background subtraction. Each MS/MS was performed with a collision-offset voltage (CE) of 30 ± 10 V and accumulation time of 50 ms for a total cycle time of 1.05 s. For SWATH acquisition, a TOF-MS scan (m/z 140−1250, 150 ms) was followed by 100 MS/MS experiments with variable Q1 windows[26] acquiring from m/z 80 to 1500 (25 ms each). The total cycle time was 2.7 s. The collision-offset voltage was set to 30 ± 5 V.

Data were acquired using Analyst TF 1.7.1 software and raw data visualized with PeakView 2.2 with Masterview 1.1 (Sciex). IDA data were searched with ProteinPilot 5.0.3 (through the cloud-based Sciex OneOmics Suite 3.1 platform) against the UniProtKB/Swiss-Prot database containing common protein contaminants (release date March 2019), with the following criteria: iodoacetamide as cysteine alkylation, trypsin for digestion and human species. Proteins and peptides were identified at a threshold of 1% global false discovery rate (FDR). The mass spectrometry proteomics data from high-resolution IDA experiments have been deposited to ProteomeXchange Consortium via the PRIDE[27] partner repository with dataset identifier PXD041752.

(Username: reviewer_pxd041752@ebi.ac.uk, Password: fZEwZ7Qd)

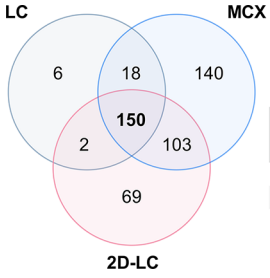
LC-MRM Analysis and Data Processing. Samples were injected using the same UHPLC model, column, and elution gradient as described above. A Sciex QTRAP 5500 (hybrid quadrupole-linear ion trap) equipped with a Turbo IonSpray ion source in positive mode was used to perform scheduled multiple reaction monitoring (sMRM), with source parameters as above. sMRM methods were built for the detection of one unique peptide per protein and two transitions per peptide. For each transition, a minimum dwell time was set to 5 ms, with a retention time window of 150 ms and total target cycle time of 1.25 s, using Analyst 1.7 software for data acquisition. Quality control (QC) samples, with an in-house standard mix of seven peptides, namely, leu-enkephalin, ACTH 4-10, fibronectin, angiotensin II, bradykinin, LH-releasing hormone, and glu-fibrinopeptide, were analyzed at the start of each sample batch and every 10 injections. Peak areas of these QC peptides (two MRM transitions each) and retention times were monitored to ensure consistent sensitivity and chromatography throughout the analysis batches, using a 12 min gradient and the same source conditions as described above. Deviations of the QC peptides were never more than 0.2 min in retention time or 15% in peak areas over the course of the analysis batches.

Target peptides were chosen based on previous LC-HRMS/MS data, while also using SRMAtlas[28] (Institute for Systems Biology) and neXtProt[29] (Swiss Institute of Bioinformatics). A few proteins of interest from the literature,[4,13,14,17,30−34] previously detected in tears, were also optimized for MRM analysis. A list of 596 protein groups was initially targeted in two sMRM methods, from which 226 protein groups were then chosen, based on their robust signals in samples from 16 volunteers. A final method compiling these 226 proteins was employed to study inter-day and inter-individual variabilities in tear samples from nine individuals on three different days. The first transition (frag 1) was used for quantitation, and the second transition (frag 2) was used as a qualitative confirmation of the peak to integrate. LC-MRM peaks were integrated using MultiQuant 3.0.2, and PeakView 2.2 (Sciex) was used for raw data visualization. Resulting quantitative data was imported into MarkerView (Sciex) to perform data normalization using the sum of all peptide peak areas and unsupervised principal component analysis (PCA). Panther software[35] was used for protein classification and gene ontology, while Reactome was used for pathway analysis.[36]

RESULTS AND DISCUSSION

Creation of an In-house Protein Database. The tear film is composed of a wide range of proteins with over 1600 proteins previously detected in tears.[13−15,17] It is estimated that around 70−85% of the tear proteome is made up of six major proteins, namely, lipocalin-1, serum albumin, lysozyme, lactotransferrin, secretory immunoglobulin A, and lipophilin;[37−41] however, certain proteins can be as low as pg/mL, with proteins secreted from cells and the ocular surface usually ranging from μg/mL down to ng/mL in tears.[3] The detection of lower abundant proteins can be quite challenging since their corresponding peptides can be masked or suppressed by co-eluting signals of higher abundance.

To increase proteome coverage, different strategies of sample preparation can be implemented prior to LC−MS/MS analysis.[10,42] From comparing three workflows, peptide fractionation by MCX-SPE or HPLC both yielded an increased number of identified proteins and higher sequence coverage compared to data acquired from unfractionated samples, especially for low abundant proteins. In Figure 1, these results



Method	# of proteins	Average sequence coverage	
		Total proteins	150 overlapping proteins
MCX	411	27%	48%
2D-LC	324	22%	35%
LC	176	24%	26%

Figure 1. Comparative analysis of the number of identified proteins (at 1% false discovery rate) in tears from a single volunteer using three workflows: 2D-LC (eight concatenated fractions), MCX fractionation (five fractions), and direct LC−MS/MS analysis of peptide digests.

were compared using tears from the same person. Whereas 176 proteins were identified by LC−MS/MS from one injection, 324 and 411 proteins resulted from the analysis of eight HPLC fractions and five MCX fractions, respectively. Interestingly, MCX fractionation identified a higher number of proteins with less fractions compared to the 2DLC−MS/MS approach. The MCX approach is also higher throughput since many samples can be processed simultaneously. Therefore, with the aim of creating an in-house spectral database of confidently identified

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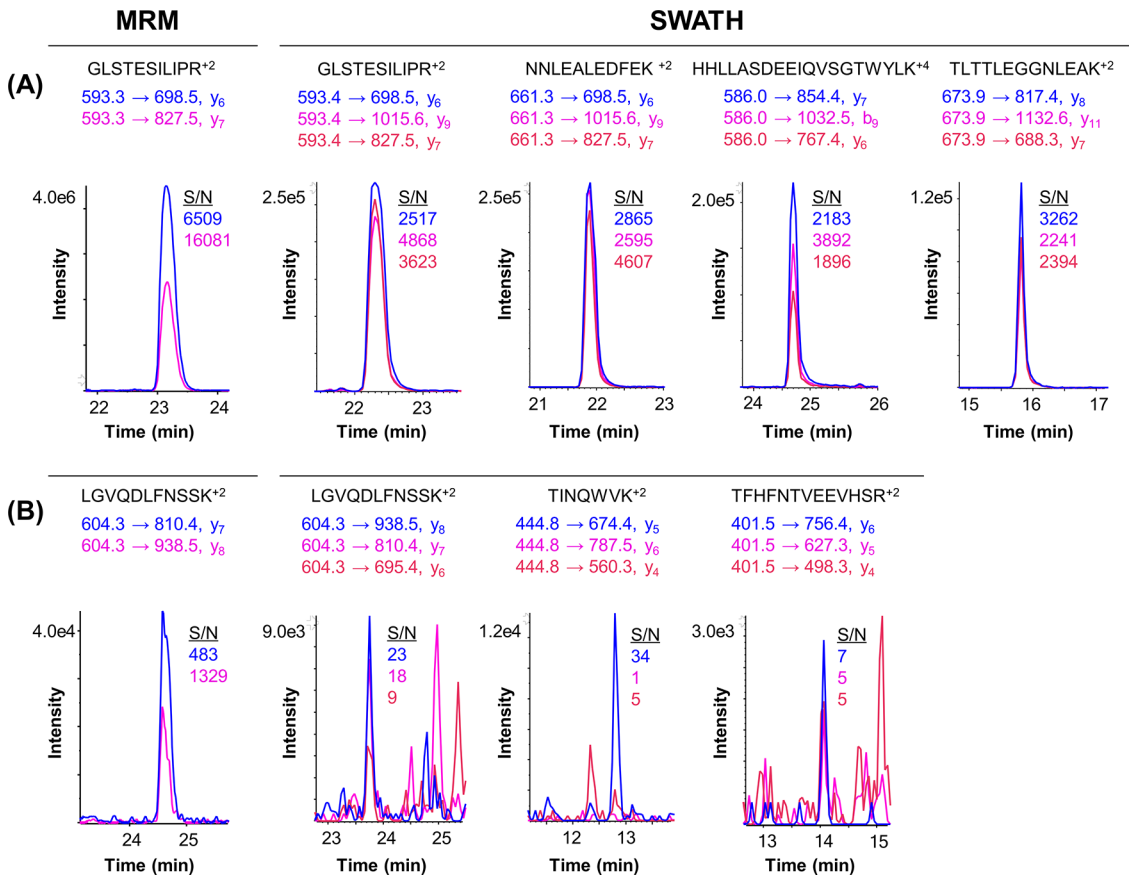

Figure 2. Representative chromatograms for peptides from (A) lipocalin-1, a highly abundant protein in tears, and (B) leukocyte elastase inhibitor, a low abundant protein, from LC-sMRM compared with LC-SWATH analyses, with signal-to-noise (S/N) values specified for each transition.

peptides from tear proteins, MCX fractionation was performed on samples from 12 different volunteers. These results were combined to those from the analysis of eight HPLC fractions from a single individual and LC−MS/MS without pre-fractionation from three volunteers for a total of 886 proteins identified in tears at a threshold of 1% global FDR with at least one peptide over 95% confidence (Table S1). Raw and processed data from these analyses have been uploaded onto Proteome Exchange via the PRIDE database and thus are accessible for subsequent studies.

Choice of Quantitative Proteomics Approach. Both data-independent (SWATH) and MRM analyses can yield quantitative data using selective fragment ions from peptides of interest. SWATH has the advantage of not needing prior method development since data is acquired in a non-biased manner. MRM, however, still offers several important advantages, including the ability for greater sensitivity, and more robust triple quadrupole instruments with much smaller resulting data files for easy data management. The most obvious difference between the two approaches can be seen in the case of low abundant proteins. Figure 2 demonstrates some representative data for two proteins, namely, the highly abundant lipocalin-1 and a much less abundant protein,[3] a leukocyte elastase inhibitor, where MRM data yields better signal-to-noise and increased robustness of measurement. For lipocalin-1, both MRM and SWATH data are reliable, whereas with the leukocyte elastase inhibitor, from the three peptides selected for quantitation via SWATH, only one peptide out of the three has an acceptable peak, while the other two contain interfering background noise. To perform accurate quantitation, only high quality and interference-free transitions should be used. During MRM method development, peptides with good chromatographic behavior and signal-to-noise can be selected for robust quantitation.

SWATH analysis is an effective screening tool and can be used for a comprehensive quantitative assessment of proteins.[43] However, the larger precursor ion windows used in SWATH become less reliable when multiple interferences exist, and therefore, a targeted MRM method is often preferred.[42] Untargeted SWATH analyses are quite useful for the discovery of putative disease biomarkers, for instance, and are often followed by more targeted analyses using LC-MRM to verify proteins of interest.[44,45]

In a multiplexed targeted method, as the number of proteins increase, so does the need for previous knowledge of peptide chromatographic retention behavior to optimize sensitivity using a scheduled MRM method. Different tools can also be used, including SRMAtlas[28] to find peptides and transitions based on previous analyses, and neXtProt[29] to choose unique tryptic peptides. Although advanced software can help, it is important to verify data quality. With the aim of studying variations in tear proteins from multiple volunteers, an optimized, targeted LC-sMRM method with verification of all peak integrations was chosen for this study.

LC-sMRM Analysis of Tear Proteins. A targeted method was developed based on the list of 886 identified proteins from data-dependent analyses, with a few extra proteins of interest compiled from the literature (Table 1). Three peptides per protein were initially selected during method development, with 596 protein groups having one unique peptide and two

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Table 1. Number of Proteins Identified by Untargeted LC-HRMS/MS and Targeted by LC-sMRM

method (# of individuals)	# of identified proteins	# of proteins optimized for LC-MRM
MCX (12)	841	547
2D-LC (10)	324 (30 unique)	2
LC (3)	240 (15 unique)	1
from the literature[4,13,14,17,30−34]		46

transitions finally being included in two LC-sMRM methods (Table S2). Following the analysis of 31 samples from 16 volunteers, a final list of 226 peptides was selected having consistently adequate signal-to-noise (>100) and peak areas (>10^4) in all samples and reliable (Gaussian-like) peak shape. These peptides were used to study the variation of their corresponding proteins in nine volunteers over three days (Table S2).

Inter-individual and Inter-day Variabilities in Tear Proteins. Tear protein composition between individuals has been shown to vary naturally depending on gender and age but also during tear collection.[18−20,24] Tears are directly exposed to the environment and can therefore be subject to several variations, involving the regulation of biological pathways in the maintenance of ocular health and homeostasis.[7] Variations can be observed between different people, as well as within the same individual, and are considered as a normal response of the ocular surface to internal and external factors. With the goal of identifying tear proteome perturbations associated with diseases, obtaining more information of the variation of proteins in tears from a non-disease cohort can help prioritize proteins in subsequent biomarker studies.

To investigate the inter-day and inter-individual variabilities of proteins detected in tears, samples were collected from both eyes of nine volunteers without any known eye disorders, on three different days. A total of 54 samples (six samples per individual) were digested and analyzed with the developed LC-sMRM method to follow the quantitative changes in 226 proteins, using each peptide peak area normalized to the sum of all peak areas from the complete method in each sample. The relative standard deviations (RSD, or %CV) were then calculated for each person (six samples per individual) as well as for each day of collection (18 samples per day). Proteins were separated into three different groups based on the range of variabilities detected for these proteins, from 15 to 105 inter-day %CV and from 22 to 216 from inter-individual %CV. Low inter-day and inter-individual variabilities were defined as having less than or equal to 30 and 40%, respectively. A protein was considered of intermediate variation between 30 and 60% for inter-day and 40 and 80% for inter-individual. Finally, >60% (inter-day) and >80% (inter-individual) variabilities were considered high. These categories resulted in 61, 111, and 54 proteins having low, medium, and high inter-day variations, respectively, whereas 49, 114, and 63 proteins were considered to have low, medium, and high inter-individual variations, respectively (Figure 3A).

Pearson correlation analysis between peak areas and variabilities (%CVs) was performed, and Figure 4A demonstrates that the variations do not correlate with the integrated peak areas. Peaks having lower abundance or signal-to-noise could be affected more severely and thus show increased variability of detection. This was also the reason that it was important to select peptides in this study that were very well detected to remove this potential bias.

The variabilities of all 226 proteins are compiled in the Supporting Information (Table S3). Larger inter-individual than inter-day variabilities were observed for all proteins measured, confirming that there was more variation between individuals than within the same person sampled on different days (Figure 3B). Most proteins also showed similar trends between inter-day and inter-individual variabilities. An unsupervised PCA plot resulting from the quantitative analysis of these 226 proteins demonstrates the overall variation seen in the 54 samples (Figure 5). It is possible to recognize from this analysis that different tear samples from a given individual group together, while others are less clustered.

As mentioned previously, all proteins exhibit higher inter-individual variabilities than the variation of the same individual on different days. This trend has also been reported in other biofluids.[46−48] Lipocalin-1 and lysozyme C are two of the most abundant proteins in tears, and both exhibited relatively low inter-day and inter-individual variabilities from this analysis. Serum albumin is also a highly abundant protein but interestingly showed a much higher variation. Upon protein classification using Panther and Reactome, certain pathways showed to be significantly enriched based on these variabilities (Figure 4B). For instance, relatively high variabilities were seen for proteins involved in glycolysis, glutathione conjugation, and the cytoskeleton. On the other hand, proteins involved in the

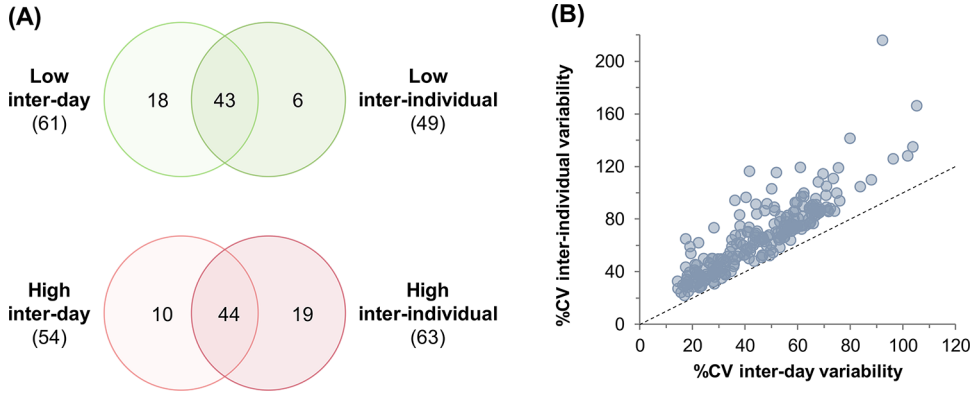

Figure 3. (A) Venn diagrams showing the number of proteins with low and high inter-day and inter-individual variabilities and (B) comparison of inter-day and inter-individual variabilities for each of the 226 targeted proteins.

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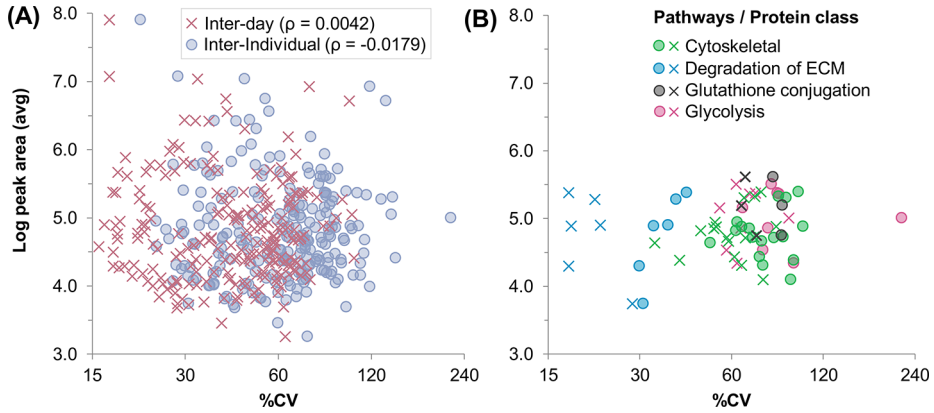

Figure 4. Correlation analysis between the normalized peak areas (A) for all targeted proteins and their associated inter-day and inter-individual variabilities and (B) for proteins implicated in cytoskeleton, glycolysis, glutathione conjugation, and degradation of the extracellular matrix.

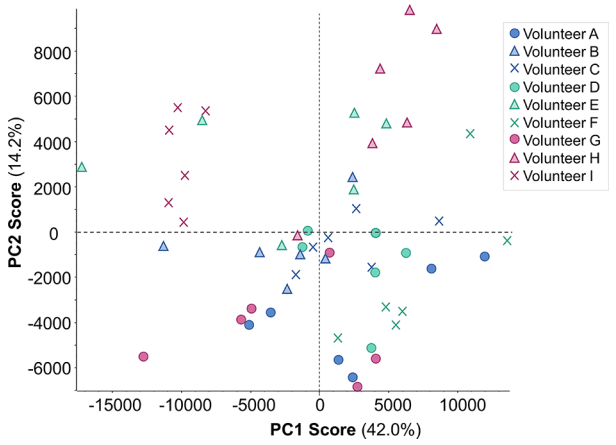

Figure 5. Unsupervised PCA plot of combined data for 226 target proteins from tear samples of nine volunteers collected on three separate days.

degradation of the extracellular matrix had significantly lower variabilities.

Glycolysis produces the main energy source to maintain the hydration level of the ocular surface.[49] From the list of 226 proteins studied here, eight are implicated in glycolysis, five of which were classified as highly variable, while the other three showed variabilities on the higher end of the intermediate category (Table 2A). The glucose level can fluctuate between the tear fluid and the interstitial space. The tear glucose content has been reported to be highly dynamic,[50] which correlates with the high variation of glycolytic proteins.

Tears play an important role in protecting the ocular surface, with different mediators being locally released.[51] Glutathione is an abundant antioxidant found in tears and is continuously regenerated to maintain homeostasis.[52] Aldo-keto reductase, cytosolic non-specific dipeptidase, and glutathione S-transferase P are implicated in glutathione conjugation and showed high inter-day and inter-individual variabilities (Table 2B), indicating the dynamic regulation in this pathway for maintaining ocular health from external stimulants and irritants in our environment.

One pathway that was specifically enriched when we examined the proteins exhibiting low variability was the degradation of the extracellular matrix (Table 2C). The metalloproteinase inhibitors 1 and 2 (TIMP1 and TIMP2), cathepsin D and B, and E-cadherin (cadherin-1) resulted in low inter-day and inter-individual variabilities. The basement membrane-specific heparan sulfate proteoglycan core protein also showed a low inter-day variability. This protein is also implicated in carbohydrate metabolism, potentially explaining why it had a slightly higher variation between individuals. The extracellular matrix is a major component of the cellular microenvironment fulfilling different functions. Abnormal dynamics of the extracellular matrix can cause deregulation of cell proliferation, death, and differentiation, leading to various pathologies. The role of metalloproteinases is to degrade the extracellular matrix to allow cell growth and facilitate remodeling. Proteolysis becomes pathological when the normal balance between proteases and their inhibitors is no longer present.[53] The low variation of these proteins indicates a good balance observed between individuals having no specific eye complaints. It also indicates that these could be very useful biomarkers if found to be specifically increased or decreased in the context of a given disease.

Cytoskeletal proteins can be separated in three sub-classes corresponding to actin (or actin-binding proteins), intermediate filament, and microtubule-binding proteins. They form a dynamic network in the cytoplasm of eukaryotic cells providing structure and support for the cell and are responsible from several functions, including cell communication, migration, and division. From our targeted list of 226 proteins, 17 were classified as cytoskeletal proteins. Half of these proteins were found to be highly variable, while the other half had medium variabilities (Table 3). Some cytoskeletal proteins were implicated in the axon guidance pathway. The neuronal growth cone of the axon detects signals in the environment via specific receptors, determining the direction of axon growth. This requires the coordination of actin filaments and microtubules.[54] The variabilities of many of these proteins reflects the dynamic nature of the cytoskeleton.

Eye diseases are widespread in the population, affecting over 2.2 billion people worldwide, often leading to vision loss,[55] as well as other side effects, such as discomfort, dryness, visual disturbance, and ocular surface damage, with significantly reduced quality of life.[56] Several studies have focused on finding protein biomarkers of different eye pathologies, such as dry eyes,[30,57−67] keratitis,[68,69] diabetic retinopathy,[70] glaucoma,[31] and keratoconus.[71−73] We found that 55 of the proteins measured in this study have previously been associated to specific eye diseases, as summarized in Table S4. Some proteins appear to be more variable than others, making those

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Table 2. Percent Variations in Proteins Involved in (A) Glycolysis, (B) Glutathione Conjugation, and (C) Degradation of the Extracellular Matrix, Detected in Tear Samples from Nine Volunteers

(A)

Protein name	Gene name	Accession number	Inter-day (%CV)	Inter-individual (%CV)
Alpha-enolase	ENO1	P06733	69	86
Fructose-bisphosphate aldolase A	ALDOA	P04075	64	79
Glucose-6-phosphate isomerase	GPI	P06744	62	96
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	P04406	72	85
Phosphoglycerate kinase 1	PGK1	P00558	55	65
Pyruvate kinase PKM	PKM	P14618	62	81
Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	PPP2R1A	P30153	58	76
Triosephosphate isomerase	TPI1	P60174	92	217

(B)

Protein name	Gene name	Accession number	Inter-day (%CV)	Intra-individual (%CV)
Aldo-keto reductase family 1 member A1	AKR1A1	P14550	72	88
Cytosolic non-specific dipeptidase	CNDP2	Q96KP4	65	88
Glutathione S-transferase P	GSTP1	P09211	66	82

(C)

Protein name	Gene name	Accession number	Inter-day (%CV)	Intra-individual (%CV)
Basement membrane-specific heparan sulfate proteoglycan core protein	HSPG2	P98160	18	43
E-cadherin (cadherin-1)	CDH1	P12830	18	30
Cathepsin B	CTSB	P07858	22	37
Cathepsin D	CTSD	P07339	21	39
Metalloproteinase inhibitor 1	TIMP1	P01033	18	33
Metalloproteinase inhibitor 2	TIMP2	P16035	28	31

Table 3. Percent Variations of Cytoskeletal Proteins Detected in Tear Samples from Nine Volunteers

Protein name	Gene name	Accession number	Protein class Panther	Biological process Panther	Molecular function Panther	Reactome pathway	Inter-day (%CV)	Intra-individual (%CV)
Actin, alpha cardiac muscle 1	ACTC1	P68032	Actin cytoskeletal			Axon guidance	84	103
Myosin light polypeptide 6	MYL6	P60660	Actin cytoskeletal			Axon guidance	71	89
Ezrin	EZR	P15311	Actin cytoskeletal		Cytoskeletal protein binding	Axon guidance	53	65
Myosin regulatory light chain 12B	MYL12B	O14950	Actin cytoskeletal		Cytoskeletal protein binding	Axon guidance	34	51
Actin-related protein 2/3 subunit 4	ARPC4	P59998	Actin cytoskeletal	Actin filament	Cytoskeletal protein binding	Axon guidance	52	69
Alpha-actinin-4	ACTN4	O43707	Actin cytoskeletal	Actin filament	Cytoskeletal protein binding		59	75
PDZ and LIM domain protein 1	PDLIM1	O00151	Actin cytoskeletal	Actin filament	Cytoskeletal protein binding		53	65
Cysteine-rich protein 1	CRIP1	P50238	Actin cytoskeletal	Cell communication			47	62
Adenylyl cyclase-associated protein 1	CAP1	Q01518	Actin cytoskeletal	Cell communication	Cytoskeletal protein binding		67	83
POTE ankyrin domain family member I	POTEI	P0CG38	Actin cytoskeletal	Localization/ Movement			75	100
Filaggrin-2	FLG2	Q5D862	Cytoskeletal				40	96
Cofilin-1	CFL1	P23528	Non-motor actin binding	Actin filament	Cytoskeletal protein binding	Axon guidance	58	71
Gelsolin	GSN	P06396	Non-motor actin binding	Actin filament	Cytoskeletal protein binding		53	63
Macrophage-capping protein	CAPG	P40121	Non-motor actin binding	Actin filament	Cytoskeletal protein binding		76	94
Profilin-1	PFN1	P07737	Non-motor actin binding	Actin filament	Cytoskeletal protein binding	Axon guidance	72	86
Tubulin alpha-1B chain	TUBA1B	P68363	Tubulin	Microtubule	Structural constituent (cytoskeleton)	Axon guidance	66	91
Tubulin beta-4B chain	TUBB4B	P68371	Tubulin	Microtubule	Structural constituent (cytoskeleton)	Axon guidance	65	76

potentially less useful as reliable biomarkers in tears related to a specific ocular disease. Normal variations in protein distribution from healthy volunteers are therefore quite useful to ascertain which proteins could serve as robust biomarkers, and therefore, this information is valuable when prioritizing clinically relevant biomarkers. This study has targeted 226 well-detected proteins in tears to study variabilities in a population with no eye disease. Though some previous studies have reported variabilities in certain proteins, this work represents a larger coverage in the tear proteome in this

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context, using a targeted LC-MRM method that can be implemented on common triple quadrupole platforms amenable to routine clinical assays.

CONCLUSIONS

A targeted scheduled LC-MRM method has been optimized to follow the variability of 226 proteins in tear samples from a group of individuals with no known eye disorders. This method has been used to study inter-individual variations but also fluctuations within the same person on different days. In the context of this study, the aim was to cover as many well-detected proteins as possible in a single targeted LC-sMRM method. A limitation of this approach, of course, is that if the peptide chosen is in a region where post-translation modifications occur, the dynamics that are measured herein involve not only the protein abundance but also potentially the level of modification. Specific pathways have been highlighted in this study, showing the high variation of proteins implicated in glycolysis, glutathione conjugation, and proteins involved in the cytoskeleton, as well as a low variation involving proteins in the degradation of the extracellular matrix. Knowledge of inter-individual and inter-day variabilities could help distinguish which proteins could be better biomarkers of eye disease, as well as help choose specific proteins of interest useful for normalization of data in subsequent studies.

This study has focused on developing a targeted method for over 200 proteins that are consistently well detected in tear samples from healthy volunteers, as well as looking specifically at their inter-individual and inter-day variations. This targeted method could be used in subsequent studies with patient samples to select proteins biomarkers of interest to specific pathologies. The ability to ascertain which proteins have greater variations without the presence of any specific eye complaints could help prioritize which proteins may be better disease biomarkers. Future work could utilize this information in the context of diagnosis and staging diseases, as well as following treatment outcomes and side effects.

ASSOCIATED CONTENT

Data Availability Statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD041752.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c03186.

(Table S1) List of 886 proteins identified in tears, compiled from multiple data-dependent LC-HRMS/MS acquisitions of fractionated and unfractionated tear digests; (Table S2) MRM transitions optimized for the targeted analysis of 596 protein groups; (Table S3) inter-day and inter-individual variabilities (%CV) of normalized peptide LC-MRM peak areas for targeted 226 protein groups measured from nine volunteers on three collection days; and (Table S4) biomarkers of eye diseases from the literature with their variabilities from this study (XLSX)

AUTHOR INFORMATION

Corresponding Author

Lekha Sleno − *Chemistry Department, Université du Québec à Montréal, Montreal, Quebec H3C 3P8, Canada;* orcid.org/0000-0001-7923-8019; Email: Sleno.lekha@uqam.ca

Authors

Maggy Lépine − *Chemistry Department, Université du Québec à Montréal, Montreal, Quebec H3C 3P8, Canada*

Oriana Zambito − *Chemistry Department, Université du Québec à Montréal, Montreal, Quebec H3C 3P8, Canada*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c03186

Notes

The authors declare no competing financial interest.

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