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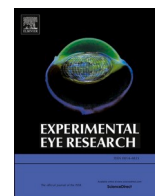
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Research article

Expanded biochemical analyses of human tear fluid: Polyvalent faces of the schirmer strip

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ABSTRACT

The tear film forms a protective barrier between the ocular surface and the external environment. Despite its small volume, recent advancements in preanalytical and analytical procedures have enabled its in-depth analysis using multiple approaches. However, the diversity of tear film collection methods and the lack of standardization in pre-analytical methods represent the main obstacles to reproducible results and comparison among different studies. In this study, we first improved the pre-analytical procedures for the extraction of various molecular entities from Schirmer strips (ScS). Subsequently, our investigation focused on analyzing the molecular variances that might occur between two primary tear collection methods: capillary tube (CT) and ScS. Additionally, we examined different parts of the ScS to underscore these variations, which could serve as crucial factors for developing a standardized, optimized protocol for sample processing. Our results show that the inclusion of surfactants in the extraction process enhanced both the yield of protein extraction and the number of proteins identified in ScS, by effectively lysing the cells and improving the solubility of several intracellular proteins. In addition to proteins, nucleic acids could also be recovered for gene expression analyses, particularly from the bulb region of the ScS which is placed in the cul-de-sac. Despite their diluted nature, extracts from ScS remain a suitable material for retrieving tear proteins such as IL-17A at levels as low as the fg/mL range, thanks to highly sensitive immunoassays. Collection methods can affect measured tear protein levels. Lactoferrin is found in higher percentages in capillary electrophoresis analysis of tears collected using ScS compared to tears collected by CT (39.6 ± 4.8% versus 31 ± 4.4%).

1. Introduction

The tear film (TF) consists of a complex mixture of proteins, lipids,

metabolites, and electrolytes, secreted primarily by lacrimal and meibomian glands and goblet cells. This crucial biofluid covers the entire ocular surface (OS), providing protection, lubrication, nutrition and an

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optically smooth surface required for proper refraction of light onto the retina (Willcox et al., 2017; Kopacz et al., 2021; Masoudi, 2022; Cwiklik, 2016; Versura and Campos, 2013). Alterations in TF components may reflect the health status of the OS and, more broadly, of the lacrimal functional unit (Masoudi, 2022; Nättinen et al., 2021; Zhou and Beuerman, 2012). Therefore, TF components represent a suitable material for the evaluation of OS diseases (OSD) and also for prognostic and diagnostic purposes (Azkargorta et al., 2017). Biochemical analysis of TF represents a promising non-invasive approach for potential use in diagnosis and monitoring of disease progression, as well as for evaluation of treatment efficacy in the future, following validation in clinical trials (Rentka et al., 2017). Despite its small volume, the TF has several advantages for biochemical analysis, development of new drugs, and potentially, biomarker investigations in various systemic diseases such as neurodegenerative disorders (e.g., Alzheimer's disease and Parkinson's disease), multiple sclerosis, and certain cancers (Bachhuber et al., 2021; Gijs et al., 2021; Król-Grzymała et al., 2022; Hamm-Alvarez et al., 2019; Boerger et al., 2019; Böhm et al., 2012).

Technological advances, especially in the field of omics, have dramatically transformed investigations thanks to extremely high detection sensitivity enabling quantification of very small samples such as tears (Hagan et al., 2016). These improvements have consequently led to an increase in the number of investigations on TF, particularly in the field of OSD (Hagan et al., 2016). The aging of the population, lifestyle changes and notable increases in the utilization of digital devices have dramatically increased the prevalence of OSD (Nättinen et al., 2021). Therefore, tear collection methods have become of major interest in order to identify candidate biomarkers in OSD such as dry eye disease (DED), using powerful “omics” technologies (Versura and Campos, 2013; Azkargorta et al., 2017; Pinto-Fraga et al., 2018; Enríquez-De-Salamanca et al., 2012; Kuo et al., 2019). Indeed, the TF can be obtained using different collection methods, such as capillary tubes (CT), Schirmer strips (ScS), absorbent cellulose acetate filters, sponges, swabs, and polyester wicks (Pieczyński et al., 2021). Different tear sampling methods are known to affect the composition of the samples and the results of tear proteome analysis (Afshari et al., 2015; Wuen Ma et al., 2021; Runstrom and Brian Tighe, 2013). Indeed, the proteomic profile of tears collected with ScS and CT has shown differences (Nättinen et al., 2020). Among tear collection methods, ScS as an indirect method and CT as a direct method represent the most commonly used approaches (Pieczyński et al., 2021; Posa et al., 2013; Ponzini et al., 2022; Winiarczyk et al., 2022). ScS are also widely utilized in omics investigations of tear fluid, including proteomics, lipidomics and metabolomics (Boerger et al., 2019; Koduri et al., 2023; Khanna et al., 2022; Catanese et al., 2023; Dor et al., 2019). Compared to CT, ScS samples collect superficial cells from the small areas of the bulbar and tarsal conjunctiva that are in contact with the strip bulb; at the same time, they collect tear fluid and contain more mucins, lipids and cellular components (Rentka et al., 2017; Afshari et al., 2015). Secreted proteins can be collected using the CT, while a combination of cellular and soluble proteins can be collected using ScS (Green-Church et al., 2008). Hence, this dual collection approach, cellular and soluble, using ScS may provide a broader range of proteins, offering a more comprehensive assessment of the OS status. Collection of tears using ScS is relatively easy, more convenient, and more rapid for patients than collection with a CT, which requires a delicate and generally time-consuming procedure with the eye open (Posa et al., 2013; Ponzini et al., 2022). In some OSD, such as DED, collection of TF with a CT is challenging. Moreover, both methods can induce reflex tearing as a consequence of resultant irritation (Dumortier and Chaumeil, 2004), foreign body sensation, and vascular plasma leakage (Nättinen et al., 2021; Posa et al., 2013; Ponzini et al., 2022). Another limitation is the lack of a standardized elution protocol (Denisin et al., 2012; van Der Meid et al., 2011). Collection with a CT is more restricted to secreted/soluble components, and this method requires trained, skillful practitioners (Ponzini et al., 2022). Despite several advantages of using ScS-extracted proteome in research,

the lack of standardized protocols for processing these samples poses challenges for analysis and inter-study comparisons (Vergouwen et al., 2023a; van Der Meid et al., 2011). Upon tear collection, various factors such as storage, extraction, handling, and analytical methods could also influence the biochemical profile of tears (Denisin et al., 2012). Therefore, all of these aspects should be considered before choosing the most suitable method, since each sampling method has its own advantages and disadvantages (Rentka et al., 2017).

This study aimed to assess the effectiveness of current techniques and possible improvements that might be made by modifying pre-analytical parameters, encompassing the entire process from sample collection to all subsequent processing steps up to analysis of the sample. Additionally, the study sought to emphasize the advantages of ScS-extracted proteome over tear proteome in research and its applicability to a variety of fields (e.g., various omics analyses for OSD and systemic diseases). Revealing variations in the proteome of two primary tear collection methods, assessing Schirmer strip-extracted proteome's potential for biochemical investigations, and refining strip sample processing will assist scientists in choosing the most suitable method for their studies. Moreover, these findings could contribute to optimizing technical processes.

2. Materials and methods

2.1. Subject selection

Schirmer strips or tears were collected from healthy volunteers (HS) and DED patients at 15–20 National Vision Hospital in Paris. The bulb region (B) of the ScS (Schirmer-plus®, Gecis; Neung Sur Beuvron, France) was carefully placed in the lower conjunctival cul-de-sac without the use of local anesthesia. Subsequently, the subjects closed their eyes, and the ScS were collected after 5 min unless the strip became fully saturated with tears sooner. The collected samples were immediately frozen at -80°C until analysis. The study was performed in accordance with the tenets of the Declaration of Helsinki and GCP, and written consent was obtained from all subjects after explaining the protocol and the scope of the study. The study was approved by the Ethics Committee, CPP-Ile-de-France (number: 2018-A02800-55). In order to investigate the impact of preprocessing parameters on the analytical results, several experimental procedures were designed to address specific research goals, which are summarized in Table 1.

2.2. Preprocessing methods for sample preparation

Different parts of the ScS, namely bulb (B), the rest of the strip (RS) and the whole strip (W) were used for protein and RNA extraction. In addition to ScS parts, dipped strips (DS) were also investigated (pieces of ScS dipped into tear fluid to absorb it). In order to compare the effect of two different buffers on total protein recovery, ScS were cut lengthwise into two identical pieces. Sample collection and preprocessing of ScS are illustrated in Fig. 1-a and b.

First, the effects of buffer volume, temperature and time on protein extraction from ScS were investigated. Briefly, 5 μL of pooled tears collected from HCs ($n = 4$) were adsorbed onto 10 mm pieces of ScS. Each piece was placed in a 1.5 mL cryotube, and a corresponding volume of 100 mM AmBic was added. Two different buffer volumes of AmBic (200 μL and 400 μL) were tested at three different time points (2, 4 and 16 h), at two different temperatures (4°C and 25°C). Each condition was performed in triplicate ($n = 3$).

Second, this study aimed to compare the efficacy of several extraction buffers, namely ammonium bicarbonate (AmBic), AmBic + Invitrolsol, phosphate buffer saline (PBS), PBS + Invitrolsol and Radio-Immuno-Precipitation Assay (RIPA), in extracting total protein from ScS samples. To ensure consistency, ScS samples were moistened to a minimum length of 20 mm prior to the extraction process. In order to circumvent variations between samples, ScS ($n = 3$) were cut vertically

Table 1
Summary of the studies performed to analyze ScS samples and tears.

| Goals | Analytical tool | Samples | Section of Schirmer strip |
|---|------------------------------|--|---------------------------|
| To determine optimal temperature, buffer volume, and elution time for extracting total protein from ScS soaked with tears | BCA protein assay | 36 DS (10 mm pieces of ScS dipped into pooled tears (n = 4)) | 36 DS |
| To compare the efficiency of various buffers on extraction yield from ScS | BCA protein assay | ScS (n = 15) | 15 W |
| To compare the number and the profile of identified proteins extracted in AmBic versus AmBic + Invitrosol from the bulbs of ScS | LC-MS/MS (timsTOF Pro) | ScS (n = 8) | 8 B |
| To explore the distinct distribution patterns of specific proteins among tears, ScS, and various segments within the ScS. | Western blot | Tears (n = 2), ScS (n = 13) | 9 W, 3 B, 3 RS |
| To quantify IL-17A protein expression in ScS extracts | Immunoassay (MSD technology) | ScS (n = 16) | 16 W |
| To compare the percentage of major proteins among tears, RS and DS samples | Capillary electrophoresis | Tears (n = 8); RS (n = 8), DS (n = 8) | 8 RS, 8 DS |
| RNA extraction and gene expression analysis from ScS | RT-qPCR | ScS (n = 20) | 4 W, 16 B, 16 RS |

into two equal parts to compare two different buffers each time (buffer 1 versus buffer 2) as shown in Fig. 1-b1. In total, 15 ScS collected from healthy subjects (HS) were used. The protein extraction efficacy of AmBic and PBS were compared with and without the addition of Invitrosol. Schirmer's strip pieces were fully immersed in the corresponding elution buffer. Each sample was analyzed with the corresponding tool after sample processing as shown in Fig. 1-c.

2.3. Molecular extraction and quantification

2.3.1. Protein extraction

Schirmer strips were divided as indicated for each preprocessing method and placed into a 1.5 mL cryotube. The corresponding volume of an elution buffer containing protease inhibitor cocktail (P2714; Sigma-Aldrich, St. Louis, MO, USA) was added, and the tubes were placed on an IKA® VXR basic Vibrax® (IKA®-Werke GmbH & Co. KG, Staufen, Germany) orbital shaker at 1500 motions/minute at 4 °C or 25 °C for 2, 4 or 16 h to determine the optimal parameters. The samples were centrifuged at 14,000 rpm at 4 °C for 5 min, and the supernatants were collected. Afterward, proteins were extracted at 4 °C for 4 h in the appropriate elution buffer for each experiment. Total protein content (TPC) was carried out using a BCA (bicinchoninic acid) Protein Assay Kit (Thermo Scientific, Pierce, IL, USA) and Spark® Multimode Microplate Reader (Tecan, Männedorf, Switzerland).

2.3.2. RNA extraction

Schirmer strips were utilized either in their entirety (W) (n = 4) or were dissected into the bulb (B) and the rest (RS) (n = 16) portions. Total RNA extraction from these samples was carried out using the NucleoSpin® RNA XS extraction kit (Macherey-Nagel, Düren, Germany), following the manufacturer's guidelines. Briefly, each sample W, B, or RS was submerged in 200 µL of the provided lysis buffer RA1,

supplemented with a reducing agent, tris-(2-carboxyethyl)phosphine hydrochloride (TCEP(•HCl)). After a 5-min incubation, the samples were vortexed, briefly centrifuged, and the subsequent steps adhered to the manufacturer's protocol. The total RNA elution volume was 10 µL. RNA yield and purity were evaluated using a NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). The total RNA elution volume was 10 µL. The quality and the quantity of RNAs were then measured using a NanoDrop detector (Thermo Scientific, Labtech, Uckfield, UK).

2.4. Protein analyses

2.4.1. Mass spectrometry

Proteomics analysis of the bulbs of 8 ScS was performed using timsTOF Pro mass spectrometry (Bruker Daltonics, Bremen, Germany). Sample collection and processing, sample preparation prior to mass spectrometry analysis, and data analysis were conducted as described previously (Akkurt Arslan et al., 2021). Briefly, ScS were collected from these two HCs in the morning and in the afternoon for two days. They were merged into two groups, with each group including 4 ScS. These bulbs were divided, and one group (4 bulbs) was extracted in 100 mM AmBic and the other (4 bulbs) in AmBic + Invitrosol (#MS10007, Invitrogen™) as shown in Fig. 1-c. The raw MS/MS data underwent analysis using Bruker Compass Data Analysis (version 5.1) and were subsequently processed using MaxQuant software (version 1.6.8.0) for protein identification (Cox and Mann, 2008). The subcellular compartment enrichment analysis of proteins was performed using SubcellularVis (Watson et al., 2022).

2.4.2. Western blot

The protein concentrations of the samples and lysates were determined using a BCA Protein Assay Kit (Thermo Fisher Scientific). Equivalent amounts of total protein samples were loaded onto 12% polyacrylamide gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad Laboratories). The resolved proteins were then transferred onto nitrocellulose blotting membranes (Bio-Rad Laboratories) and blocked with a 5% skim milk solution (Sigma-Aldrich, Steinheim, Germany) for 1 h. The primary antibody, beta-actin (#8H10D10), was obtained from Cell Signaling (Danvers, MA, USA), and the Actinin-4 (#E-AB-1475) and Nucleobindin-2 (#E-AB-64290) were purchased from Elabscience (Houston, TX, USA). Following overnight incubation with the appropriate primary antibodies, the membranes were incubated with secondary antibodies for 1 h.

2.4.3. IL-17A immunoassay

The level of human IL-17A was measured using Mesoscale Discovery (MSD) S-PLEX® (#K151C3S-1, Meso Scale Diagnostics, Rockville, MD, USA) according to the manufacturer's directions. The whole ScS was extracted in 400 µL of 100 mM AmBic for 4h at 4 °C for this analysis. The final concentration of each sample was normalized to 300 µg/mL for the analysis.

2.4.4. Capillary electrophoresis

Tear fluids and ScS samples were collected from both eyes of the four HS. First, 10 µL of tear samples from each eye were combined with 190 µL of 100 mM AmBic buffer, following the procedure described for capillary electrophoresis in Fig. 1-c. Subsequently, 10 µL of tears from each eye of each participant was placed in a 1.5 mL cryotube, and a 10 mm piece of ScS was dipped in the tears (10 µL) for absorption (DS). After the tears were absorbed by the ScS, 200 µL of AmBic was added to each tube for the protein extraction. Similarly, the rest of the strip (RS) was separated from the bulb to remove cellular components and then eluted in 400 µL of 100 mM AmBic. The protein extraction was conducted as shown in Fig. 1-c (capillary electrophoresis at 4 °C, for 4 h). The total protein content (TPC) was assessed using the BSA Protein Assay and subsequently normalized for all samples. The

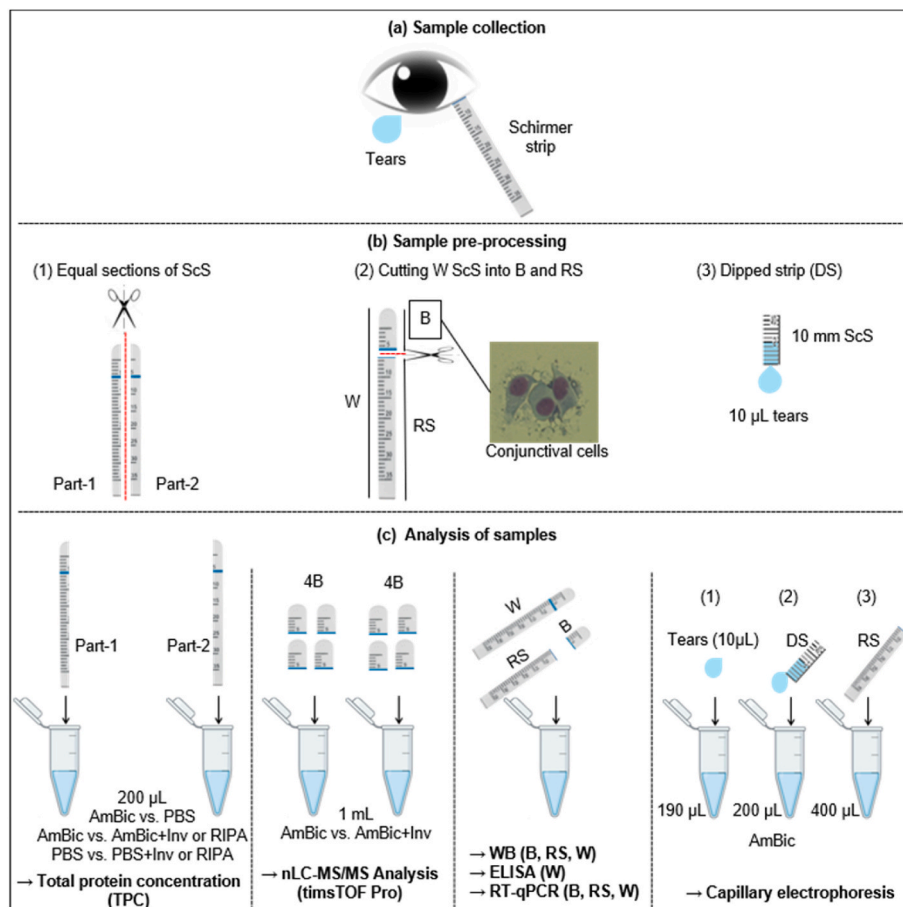


Fig. 1. Workflow of preprocessing and analytical methods for Schirmer strips (ScS). (a) Illustration of tears and ScS sample collection. (b) Preprocessing of ScS. (b-1) Strips were cut vertically into two equal parts to compare the protein extraction efficacy of two different buffers. (b-2) Whole strip (W) was divided into the bulb (B) and the rest of the strip (RS) to investigate both parts separately. The conjunctival cells attached to the bulb are shown with 400 \times magnification after May-Grünwald-Giemsa staining. (b-3) A piece of the strip was dipped into 10 μ L of tears to investigate the major tear proteins after the extraction process. (c) ScS sample preparation for various analytical techniques. ScS, Schirmer strips; W, whole Schirmer strip; B, bulb; RS, rest of the strip; DS, dipped strip; AmBic, ammonium bicarbonate; Inv, Invitrosol.

electropherogram of the samples was obtained using the gel electrophoresis system Hydrasys Hyrys® (Sebia, Lisses, France) with 10 μ L of each sample being deposited into the gel.

Furthermore, the total protein content of 10 μ L tears (T) was compared with that of 10 μ L tears absorbed on a 10 mm strip and subsequently extracted using AmBic buffer (DS) to calculate the percentage of total protein recovery from DS.

2.5. Gene expression analysis

cDNA was synthesized from equal amounts of RNA using Multiscribe reverse transcriptase (TaqMan Reverse Transcription Reagents, Applied Biosystems, Life Technologies). The concentrations of each DE sample were adjusted to 2.5 ng/ μ L of cDNA. The reaction mixture containing 12.5 ng of cDNA per well was preheated at 95 $^{\circ}$ C for 10 min, followed by 40 cycles (95 $^{\circ}$ C/15 s and 60 $^{\circ}$ C/1 min). Target cDNA was amplified using the QuantStudio™ 5 Real-Time PCR System (Applied Biosystems™, Life Technologies) with assays-on-demand primers (TaqMan probes) for human GAPDH (Hs02786624.g1) and HLA-DRA (Hs00219575_m1).

2.6. Statistical analyses

All experiments were performed at least three times. The Student's t-test was used to compare differences between the two groups. The one-

way ANOVA test was used to make comparisons among three or more groups, followed by Dunnett's post-hoc test. $P < 0.05$ was considered statistically significant. GraphPad (Version 9, GraphPad Software, La Jolla, CA, USA) was used with a risk set at 0.05 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3. Results

3.1. Impact of surfactants on the yield of the protein extraction process

Our results showed that the addition of surfactants to classical extraction buffers such as AmBic or PBS, or using special buffers containing a combination of surfactants such as RIPA, significantly increased the quantity of extracted proteins from ScS. The comparison of two conventional buffers, PBS and AmBic, did not show a significant difference (Fig. 2-a). The addition of RIPA and the MS-compatible surfactant, Invitrosol, significantly increased the quantity of extracted protein in both buffers (Fig. 2-b/2-c). The utilization of these surfactants resulted in a 95.7 \pm 28.5% increase in total protein extraction (with Invitrosol) and a 141.4 \pm 26.7% increase (with RIPA) when added to AmBic. Likewise, incorporating Invitrosol and RIPA into PBS led to a 132.4 \pm 36.1% and 292.4 \pm 76.8% increase in total protein extraction, respectively.

In this analysis, each comparison utilized different ScS with varying moistened lengths ($n = 3$). Consequently, the concentration of total

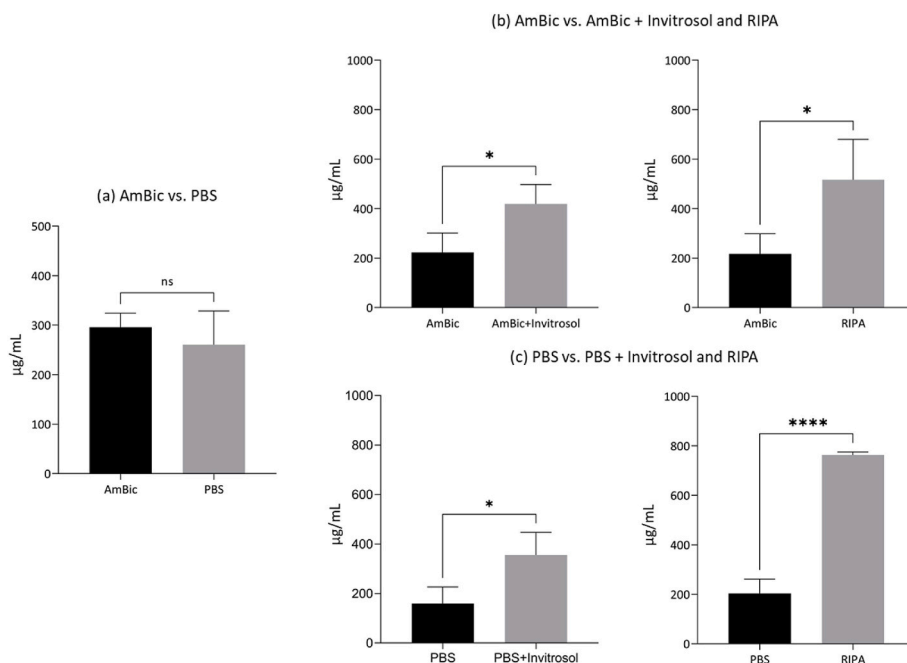


Fig. 2. Impact of different extraction buffers on the yield of total protein recovery (µg/mL). (a) Comparison of Ammonium bicarbonate (AmBic) with phosphate buffer saline (PBS) without addition of any surfactants. (b, c) Addition of MS-compatible surfactant, Invitrosol, increased the yield of extracted proteins in both AmBic and PBS. RIPA showed superiority over both classic buffers, AmBic and PBS, extracting a higher quantity of proteins (n = 3). These experiments compared two different buffers each time on Schirmer strips that were cut into two identical sections vertically. The data are shown as mean ± SD.

protein extracted using the same buffers (PBS, AmBic, and RIPA) varied across the different analyses.

Moreover, the results that compared different buffer volumes (200 or 400 µL), extraction temperatures (4 °C or 25 °C), and time (2, 4 or 16 h) showed no significant differences for protein extraction from a 10 mm section of ScS (data not shown). Slightly more proteins could be extracted at 4 °C using 400 µL of the buffer (Fig. S1).

3.2. Analysis of ScS-extracted proteome with various protein analytical tools

3.2.1. Invitrosol™ increases the number of proteins identified in the bulb

A total of 1153 proteins were identified when one of the rationally grouped four bulbs was extracted using the AmBic buffer. However, when another group of four bulbs was extracted using AmBic + Invitrosol, the number of identified proteins increased to 1883. The addition of Invitrosol to the AmBic buffer resulted in a 63.3% increase, corresponding to an additional 823 proteins identified in the MS analysis (Fig. 3). Gene Ontology (GO) cellular component analysis revealed that a large number of these proteins identified with the addition of Invitrosol consisted of membrane-bound organelle proteins (GO:0043227).

Subcellular compartment enrichment analysis of these proteins revealed that the majority of these proteins were localized in the cytoplasm (n = 757) and subcellular organelles such as mitochondria, lysosomes, endoplasmic reticulum and intracellular vesicles. These data suggest that the addition of Invitrosol to AmBIC buffer increased the identification of intracellular proteins in ScS.

3.2.2. Identification of major tear proteins in ScS compared to tear fluid

Further investigation of proteins identified on MS analysis using the Western blot technique confirmed the differential distribution of cellular proteins, such as β-actin and actinin-4 (ACTN4). These two cellular proteins were not detected in the tear sample collected using the CT (Fig. 4-a, T1, T2). They were detected in greater quantity in the bulb (B) or the whole strip (W) than in the rest (RS). Unlike tears collected by the CT, these proteins could also be detected in the rest of the strip at a lower level (Fig. 4-b). However, inter-sampling variation can be observed among the bulbs as well, as some may collect more cells than others (Fig. 4-b). The extraction buffer needs to be adjusted according to the moisture length of the ScS to obtain sufficiently concentrated samples for Western blot analysis (Fig. 4-c). In this study, 200, 300 and 400 µL of elution buffer were chosen for the ScS moistened at ≤10 mm, between

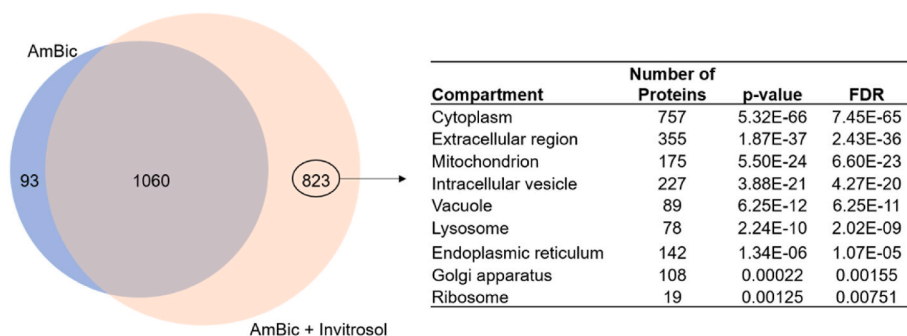


Fig. 3. Numbers of identified proteins in the bulbs extracted in “AmBic” (n = 4) and “AmBic + Invitrosol” (n = 4). The Gene Ontology Cellular Compartment enrichment analysis of the proteins identified with the addition of Invitrosol is shown in the associated table.

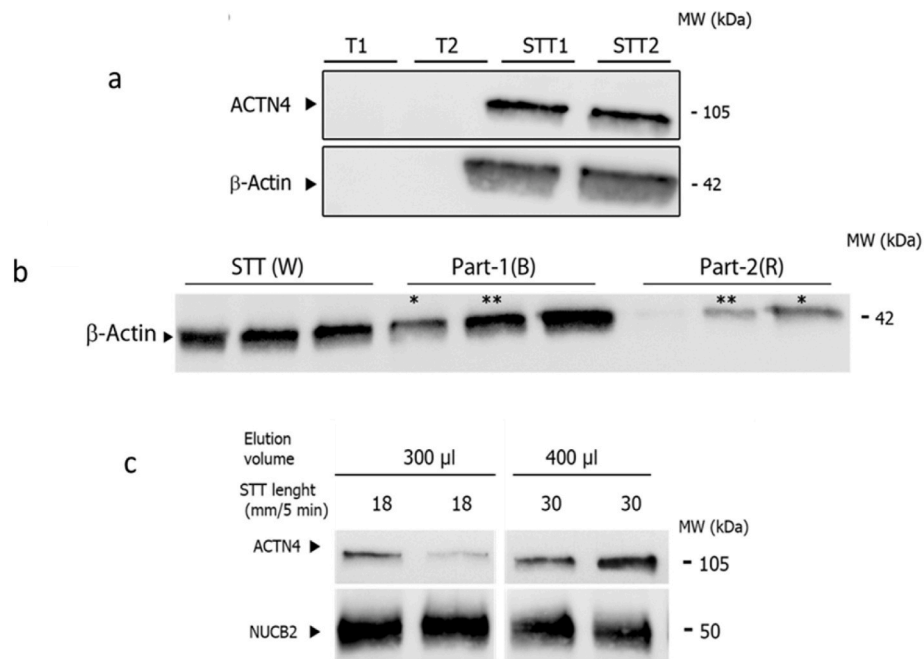


Fig. 4. Tear protein analysis from different parts of the ScS and capillary samples. (a) The housekeeping protein β -actin or another cellular protein Actinin-4 can be used as standards in the ScS-extractions (STT1, STT2), while this is not possible for tears collected using microcapillary tubes (T1, T2). (b) Higher levels of β -actin were obtained from the bulb (B) and the whole (W) strip compared to the rest (RS) of the ScS, (protein quantity normalized). (c) Normalized protein quantity of ScS with the same moistened lengths exhibited different quantities of intracellular protein ACTN4. Levels of proteins such as NUCB2 show less variability among different samples. According to the score of the Schirmer strip, the extraction volume is modified for these samples. *, Subject-1, **, Subject -2.

10 and 20 mm and ≥ 20 mm, respectively, to avoid a high variation in the concentration of the samples. The number of cellular proteins, such as ACTN4, may vary more among different ScS samples, depending on the number of cells collected, while proteins found in both extracellular and intracellular compartments, such as NUCB2, appeared in similar quantities in all samples (Fig. 4-c).

3.2.3. Cytokine detection using meso scale discovery (MSD) immunoassay

In order to explore the detectability of low-abundance proteins in the extracts of ScS, IL-17A was selected for investigation in both HS and patients with ocular surface disease (OSD). The concentration of IL-17A was 8.3 ± 3.2 (mean \pm SEM) fg/mL in HS and 67 ± 16.6 (mean \pm SEM) fg/mL in patients with OSD (Fig. 5). Such a sensitive technique qualifies ScS-extracted proteome to be a robust alternative to tear fluid for tear film cytokine investigations. Variation was observed among both patients with OSD or DED and HS groups, but the patient groups exhibited significantly higher expression of IL-17A compared to the HS.

3.2.4. Capillary electrophoresis: differential expression of lactoferrin and lysozyme in ScS-extracted proteome compared to tears

In this study, capillary electrophoresis was used to compare the profiles of major tear proteins used to assess patients for OSD in the clinic. The percentages of these major tear proteins (e.g., lactoferrin (LTF), lipocalin (LIPOC), lysozyme (LYZ)) in the samples that were obtained by (1) direct capillary tears (T), (2) proteins extracted from the RS (to exclude cellular proteins), and (3) proteins extracted from a DS (a piece of ScS dipped into tears) were compared (Fig. 6-a). Extraction from RS exhibited a significantly higher level of LTF ($39.6 \pm 4.8\%$) compared to DS ($30.4 \pm 4.4\%$) and T samples ($31 \pm 4.4\%$). Similar levels of LTF in the extraction of DS and T suggest that LTF can be recovered from ScS almost entirely. The higher level of LTF in the RS may be a result of reflex tearing caused by the ScS (Fig. 6-b). The percentage of LYZ was lower in RS ($15.9 \pm 3.1\%$) and DS ($17.5 \pm 3.4\%$) compared to T ($19.5 \pm 4.5\%$) suggesting incomplete recovery after the extraction process due to

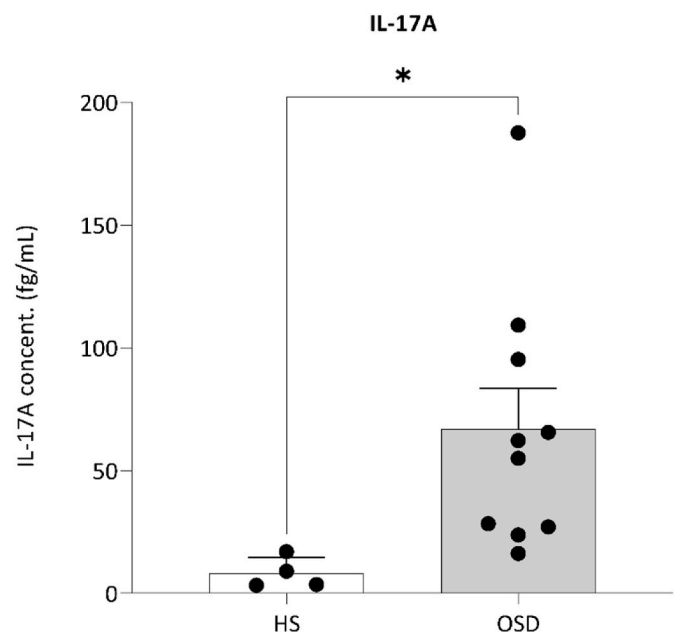


Fig. 5. Detection of IL-17A in the ScS-extracted proteins (SEP) of healthy subjects (HS, n = 4) and patients with ocular surface disease (OSD, n = 10). Highly sensitive immunoassay enabled the detection of IL-17A even in healthy subjects within the fg/mL range. The data are shown as mean \pm SEM.

retention in the strip (Fig. 6-c). Lipocalin (LIPOC) levels did not change significantly among different samples ($29.5 \pm 10.5\%$, $28.5 \pm 7.9\%$, and $30.4 \pm 10.7\%$ in T, RS, and DS, respectively) as shown in Fig. 6. Furthermore, our results revealed a recovery rate of $92.1\% \pm 5.6$ (n = 6) for total protein by comparing the total protein content of 10 μ L tears (T)

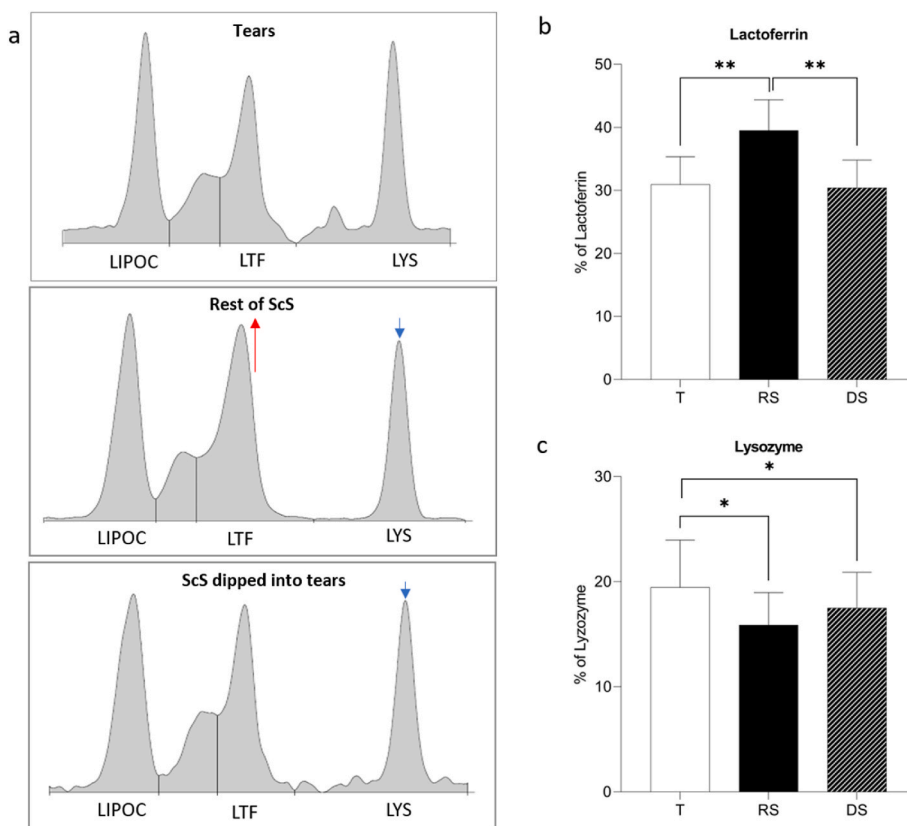


Fig. 6. Percentages of major tear proteins lipocalin (LIPOC), lactoferrin (LTF), and lysozyme (LYZ) in the tears (T), in extracted rest of the ScS (RS), and dipped strip (DS) (n = 8 eyes). The data are shown as mean ± SD.

with that of 10 µL tears absorbed on a 10 mm strip (DS).

3.3. Total RNA content and gene expression analysis in ScS-extracted proteome

Gene expression analysis from ScS was performed using the house-keeping gene GAPDH, which serves as a reference gene for normalization. Additionally, one of the commonly studied genes in OSD, HLA-DRA, was included in the analysis to assess its expression levels in the ScS samples. A higher quantity of total RNA could be recovered from the bulbs of the ScS compared to the rest of the strip or the whole strip (Fig. 7-a). The cycle threshold values of the GAPDH and HLA-DRA genes was lower in the bulb compared to the rest of the strip and the whole strip (Fig. 7-b).

4. Discussion

ScS is a highly valuable sample collection method with the ability to gather a wide range of biochemical compounds, including proteins, RNA, lipids, and others to be used in various analyses to evaluate the status of the OS. Despite the multiple advantages of ScS as a sampling method, currently, there is no standardized protocol for pre-analytical processing. Consequently, numerous studies have been conducted to explore the implications of pre-analytical sample handling and the impact of various factors on the efficiency of protein recovery for the optimization of extraction procedures from ScS, particularly for protein extraction (Denisin et al., 2012; van Der Meid et al., 2011; Vergouwen et al., 2023a; Aass et al., 2015; Krajcikova et al., 2022; Green-Church et al., 2010; Vergouwen et al., 2023b; Gijs et al., 2023). The lack of a standard protocol has led to the utilization of a wide range of extraction

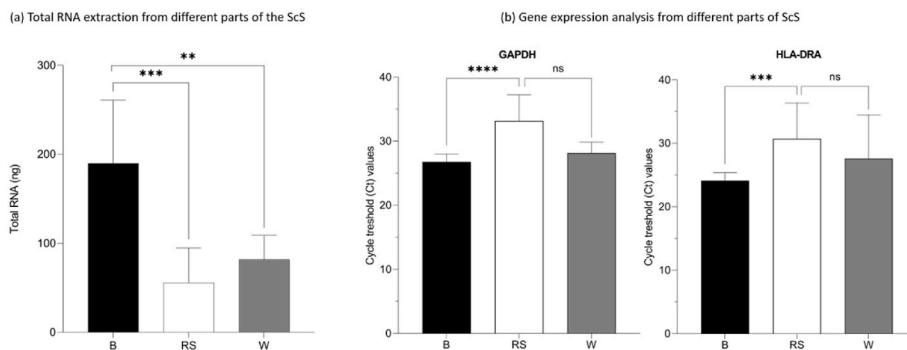


Fig. 7. RNA extraction and gene expression analysis from ScS. (a) Total quantity of RNA extracted from different parts of the Schirmer strips (n = 16 for Bulb (B), n = 6 for the rest of the ScS (RS), and n = 4 for whole strip (W)). (b) Cycle threshold detection of GAPDH and HLA-DRA transcripts from total RNA extraction in different parts of the ScS and whole strip. Data are shown as mean ± SD.

buffers in different studies to extract proteins or to compare the efficiency of these buffers. In addition, in testing various buffers, some studies have investigated other parameters that impact extraction yield (e.g., buffer volume, temperature, elution time) to determine the most convenient pre-analytical methods. In these studies, either a single extraction buffer (e.g., distilled water, sodium chloride (NaCl) solution, phosphate buffer saline (PBS), ammonium bicarbonate (AmBic), Tris buffer, or urea) or a combination of these buffers with or without the addition of various surfactants (e.g., RapiGest SF, InvitrosolTM, PPS Silent® Surfactant, Tween-20, Triton X-100, and NP-40) as well as other molecules (e.g., dithiothreitol and (thio)urea) have been used to enhance protein extraction (Bachhuber et al., 2021; Pieczyński et al., 2021; Winiarczyk et al., 2022; Denisin et al., 2012; van Der Meid et al., 2011; Vergouwen et al., 2023a; Akkurt Arslan et al., 2021; Aass et al., 2015; Krajcikova et al., 2022; Zhou et al., 2012; Ilnatko et al., 2013; Z. Huang et al., 2018; Winiarczyk et al., 2018; Miyake et al., 2018; Green-Church et al., 2010; Aqrawi et al., 2017; Chen et al., 2007).

The protein extraction process from strips is dependent on several different factors such as molecular weight and surface hydrophobicity of the proteins (Denisin et al., 2012). The solubility of proteins is highly dependent on the main physicochemical properties such as pH and composition of the extraction buffers, which may include salts, phosphates, detergents, ampholytes, chaotropic agents and reducing agents such as dithiothreitol and β -mercaptoethanol, (Ngoka, 2008). Hence, some studies use urea or its combination with thiourea to enhance protein solubility as urea disrupts hydrogen bonds, facilitating protein unfolding and denaturation, while thiourea reduces hydrophobic protein interactions (Rabilloud, 1998; Komatsu, 2015; Ngoka, 2008). Surfactants are used to enhance protein solubility in various lysis buffers. Hence, commercial MS-compatible surfactants have been developed to solubilize hydrophobic proteins and improve the in-solution protein digestion, leading to increased protein identification (Chen et al., 2007). This study assessed the impact of Invitrosol, a MS-compatible surfactant, on the efficiency of protein extraction from ScS samples and subsequent protein identification from the bulb region of the ScS containing cells. We aimed to understand how Invitrosol affects protein extraction and identification, specifically focusing on its role in the lysis of cells. Radio-Immuno-Precipitation Assay (RIPA) buffer, is one of the most effective and widely used lysis buffers to extract proteins from cells and tissues. It includes 50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS (Ngoka, 2008). RIPA likely outperforms Invitrosol in lysing conjunctival cells on the strip, as it recovers more total protein. RIPA's higher ionic strength, due to NaCl, enhances protein solubility (Mao et al., 2012). This study shows that surfactants increase total protein extraction and identification, especially intracellular proteins in MS-based analysis. Different ScS segments may exhibit diverse molecular profiles, requiring distinct extraction treatments (Akkurt Arslan et al., 2021). The bulb contains conjunctival epithelial cells, necessitating surfactant use to release membrane and cellular proteins, enhancing proteome coverage (Shen et al., 2018).

Beyond optimizing elution buffers, several studies have explored factors affecting total protein extraction yield, including buffer volume, temperature, and elution time (Denisin et al., 2012; Aass et al., 2015; Gijs et al., 2023; Chong et al., 2010; Wong et al., 2011). Research indicates protein extraction reaches maximum efficiency within 3 h (Denisin et al., 2012; Chong et al., 2010; Wong et al., 2011). Our study confirms that 4 h is sufficient, with no significant difference between 4 °C and 25 °C. Recent work found 400 μ L as the optimal buffer volume (Gijs et al., 2023), aligning with previous findings. Together, these studies propose 400 μ L and 4 h extraction as suitable parameters for optimal ScS protein recovery. Additionally, in-strip protein digestion boosts protein identification in tear samples, outperforming earlier methods (Jones et al., 2022). Furthermore, using in-strip protein digestion on ScS, a higher number of proteins were successfully identified. This approach is notable for its substantial improvement in protein identification compared to previous methods (Jones et al., 2022;

Harkness et al., 2023).

Upon successful extraction, there are variety of techniques that can be used to analyze tears and ScS samples, such as gel electrophoresis Hydrasys Hyrys®, enzyme-linked immunosorbent assay (ELISA), multiplex bead analysis and mass spectrometry for proteomic analysis, as shown in Table 2. Indeed, ScS-extracted proteome has been analyzed instead of tears in numerous studies using multiple analytical techniques for the detection and quantification of tear proteins (Wuen Ma et al., 2021; Posa et al., 2013; Ponzini et al., 2022; L. Huang et al., 2003; Magny et al., 2022; Miyake et al., 2018).

ScS-extracted proteome has been extensively analyzed using various MS-based proteomic strategies (Li et al., 2014; Nichols and Green-Church, 2009; Q. Qingyu Liu et al., 2017; Grus et al., 2005; Boehm et al., 2013; Srinivasan et al., 2012; Zhou et al., 2009) and other proteomic approaches from traditional two-dimensional gel (Soria et al., 2013; Grus et al., 2022; Versura et al., 2010) to highly sensitive immunodetection methods (Enriquez-de-Salamanca et al., 2010; LaFrance et al., 2008; Soria et al., 2017) for detection of proteins and biomarker investigations on the OS. Therefore, the detection of all proteins present in ScS samples is essential to visualize the entire landscape of molecular signatures in the OS. In addition to MS-based proteomics analysis, various other complementary proteomic approaches, which cannot entirely replace each other, can be used for ScS-extracted proteome analysis (Westermeyer, 2016). In our study, the proteins in the bulb of the strip were identified using a shotgun proteomics approach to compare the effect of adding Invitrosol on the number and composition of proteins identified. The addition of MS-compatible commercial surfactants, such as Invitrosol, significantly increases the number of identified proteins by providing access to hundreds of intracellular proteins. Indeed, extraction of these intracellular proteins, such as membrane proteins, is difficult due to their hydrophobic nature and requires the use of solubilizing agents such as surfactants or high pH solvents (Patrick and Arthur, 2019; Brun and Couté, 2019).

ScS-extracted proteome finds utility in biomarker investigations through classic immunoassays like enzyme-linked immunosorbent assays (ELISA), the preferred method for protein quantification. Yet, not all proteins have commercial ELISA kits. In such cases, Western Blot (WB) offers a reliable, cost-effective method for relative protein quantification (You et al., 2012). WB's limitations include reflecting protein concentration inaccurately, compounded by tear samples lacking a standardized reference protein (Mahmood and Yang, 2012; Willcox

Table 2

Examples of various protein analyses of ScS-extracted proteome (SEP) for ocular surface disease investigations.

| Application | Goal | Reference |
|--|---|---------------------------|
| One-dimensional gel electrophoresis (1DGE) | To analyze electrophoresis patterns of substance P in samples collected from healthy subjects | Markoulli et al. (2017) |
| Two-dimensional gel electrophoresis (2DGE) | To profile tear protein expression of dry eye and MGD patients in comparison to healthy controls | Soria et al. (2013) |
| ELISA | To compare tear concentrations of IgE with serum IgE concentrations in subjects with Japanese cedar pollinosis | Ono et al. (2005) |
| Multiplex ELISA | To measure soluble factors (e.g., IL-1 β , IL-6, IL-17A, TNF α , IFN α / β / γ , EPO, TGF β 1, and IgE) in keratoconus patients and healthy controls | D'Souza et al. (2021) |
| Antibody Microarray | To analyze proinflammatory cytokines in DED patients | Boehm et al. (2011) |
| Multiplex proteomic technologies | To measure levels of inflammatory proteins | (Vergouwen et al., 2023a) |
| Mass spectrometry | To determine proteins modulated in DED patients | Tong et al. (2011) |
| Point-of-Care Immunoassay | To measure the level of Matrix Metalloproteinase-9 in DED patients | Kang et al. (2022) |

et al., 2017). To mitigate this, an external loading control could be used for tear proteins in WB. For ScS samples, WB investigations detect abundant intracellular proteins like beta-actin and actinin-4. However, this study shows intracellular protein intensity varies across samples and strip sections, indicating varying cell collection. An external standard with dual intracellular and extracellular localization, like NUCB2, could serve as a better standard in ScS-extracted proteome studies. Additionally, research for the identification of proteins that exhibit consistent levels in tear fluid remains an unmet need. Therefore, the collaborative efforts of international researchers should be seriously considered as a means to achieve more substantial progress toward the standardization of tear fluid analysis.

Highly sensitive immunoassays have enabled reproducible and reliable detection of proteins down to the fg/mL range. Indeed, ScS proteins are diluted in the extraction buffer; hence, they are present at lower concentrations than tear proteins obtained by CT. Consequently, the detection of some proteins in ScS might be challenging using classical methods such as WB or ELISA. The concentration of IL-17A that is increased in OSD and that found in healthy individuals typically falls within the pg/mL range (Tan et al., 2014; R. Rongjun Liu et al., 2017). Since ScS-extracted proteome is rather dilute compared to tears, the level of IL-17A likely drops into the fg/mL range. Therefore, we chose to use an immunoassay to evaluate the detectability of IL-17A in ScS-extracted proteome. Indeed, the cytokine, IL-17A, could be detected in the ScS-extracted proteome of both DED patients and healthy controls using this highly sensitive assay. These innovative immunodetection assays enable the simultaneous quantification of over a thousand proteins at low concentrations down to the order of fg/mL, making it possible to detect almost any protein from the extract of the ScS (Ren et al., 2021).

Another analytical technique that has been used in tear protein analysis is capillary electrophoresis. This technique is used for the early diagnosis and prevention of DED. The major advantage of this method is its ability to identify and relatively quantify many proteins that can be informative regarding contact lens tolerance (e.g., lipocalin) or inflammation at the level of the ocular surface (e.g., immunoglobulins) (Chiva, 2011; Glasson et al., 2009; Labbé et al., 2007). Comparison of major tear protein profiles of capillary tears (T), extraction of the RS (to exclude cellular proteins), and a DS revealed significant differences in the percentage of LTF and LYZ. The higher level of LTF in the RS may be a result of reflex tearing caused by the ScS. Conversely, lower LYZ percentages in RS and DS than in T underscore its retention in the strip post-extraction. LYZ retention in ScS reaches around 15%, leading to loss after elution (Denisin et al., 2012). Our study emphasizes that major tear protein percentages differ between ScS and capillary tears, even after discarding the strip's bulb to eliminate cellular proteins. Notably, other tear proteins might also be retained in the strip, influenced by extraction buffer characteristics. These findings underline the need for a standardized extraction protocol due to substantial variations in protein elution based on the buffer employed. Standardization is essential for trustworthy comparisons across diverse studies.

The two sections of the ScS, the bulb and RS, exhibited differences in RNA extraction as well. mRNAs are molecules that can be extracted from ScS along with the proteins. Conjunctival imprints have been used for gene expression analyses to identify diagnostic biomarkers in OSD (Liang et al., 2019; Kessal et al., 2018; Hagan, 2017). However, ScS can be a good alternative for gene expression analysis and might be more convenient for patients than conjunctival imprints. Detection of mRNA in the RS, which theoretically does not contain cells, might suggest the presence of extracellular mRNA in the tear fluid. The quantity of eluted nucleic acid such as 5, 2.5, or even 1.25 ng of total RNA was sufficient to perform gene expression analysis, including housekeeping genes (data not shown). Despite normalization of the total RNA quantity for transcript analysis, the analyzed genes were more abundantly detected in the bulb compared to the RS. These data suggest that either the proportion or quality of the extracted mRNA is higher in the bulb, or a larger

proportion of mRNA molecules in the RS consists of non-coding RNA, such as microRNAs. These RNA molecules in the RS might stem from tear extracellular vesicles, as they contain sequencable RNA and prominent microRNAs (Pucker et al., 2022). The level of extracellular RNAs should be investigated further in the ScS-extracted proteome, as these communication molecules have been suggested to be biomarkers in some diseases, such as OSD and cancer (Wu et al., 2022; Wei et al., 2020). When mRNAs from conjunctival cells are of interest, considering only the bulb of the ScS is recommended.

Despite the extensive use of ScS-extracted proteome in diverse biomedical biomarker studies, a lack of standardized preprocessing methods persists. Alongside factors affecting protein recovery, such as storage temperature, ScS wet-dry storage, and manufacturing specifics (Gijs et al., 2023; Mann et al., 2018), discrepancies in absorption rates and protein extraction behavior emerge due to varying ScS shapes, thicknesses, fiber densities, and pore sizes (Gijs et al., 2023). Without standardized methods in manufacturing, storage, and sample processing, comparing and interpreting results across studies remains challenging. Establishing unified standards across strip manufacturing and analytical steps is critical for reliable, unbiased analytical outcomes (Kirwan et al., 2018). Implementing manufacturing regulations by healthcare authorities or expert group recommendations is crucial. This need stems from ScS dual usage in sample collection and clinical evaluation, with a Schirmer's score of <5 mm/5 min diagnosing dry eye disease (Posa et al., 2013; Miyake et al., 2016; Brott and Ronquillo, 2021). A unified, standardized protocol is essential to yield robust, reproducible data from ScS investigations.

5. Conclusions

Schirmer strip represents a suitable tool for collecting protein and RNA samples to be used for various investigations regarding ocular surface and systemic diseases. In this study, we provided insights into the analyses of the molecular content of Schirmer strips. These findings highlight the significance of the dual secretory and cellular composition within the strips. Depending on the specific molecular targets, it is essential to adapt analytical tools accordingly, focusing on the appropriate section of the Schirmer strip.

Finally, despite their diluted nature, the proteome extracted from Schirmer strips can be used throughout biomarker investigations, thanks to very sensitive detection tools. Advanced immunoassay technology and robust workflow for the preprocessing of ScS might enable the development of novel point-of-care devices. However, the lack of standardization in sample preparation remains a major obstacle to overcome.

Author contributions

Conceptualization: M.A.A., K.K., F.B.-B. and C.B.; methodology, M.A.A., K.K., S.C., C.P., F.B.-B., G.R., M.G.P., N.H., A.K., A.R.G.; validation, K.K., M.A.A., F.B.-B., S.C., C.P. and C.B.; formal analysis, M.A.A., K.K., F.B.-B. and C.B.; writing—original draft preparation, M.A.A., K.K. and F.B.-B.; writing, review and editing, M.A.A., K.K., F.B.-B., A.R.G. and C.B. visualization, M.A.A., K.K. and F.B.-B., supervision, K.K., M.A.A., F.B.-B. and C.B. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.exer.2023.109679>.

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