TITLE:

Phenol Red Thread-based Sampling Method for Untargeted Tear Fluid Lipidomics in Biomarker Discovery

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SUMMARY:

This protocol describes a novel clinical protocol for collecting human tear fluid samples using phenol red threads liquid chromatography-mass spectrometry (LC–MS/MS)-based workflow to discover of tear lipidomic profile. The simple methyl tert-butyl ether (MTBE)/methanol biphasic separation method enables rapid tear lipid extraction with high recovery for tear biomarker discovery.

ABSTRACT:

Tear lipids are crucial for tear film stability and ocular surface health. Changes in tear composition could be associated with ocular and systemic diseases such as meibomian gland dysfunction and dyslipidemia. Profiling tear lipids may help biomarker discovery for disease diagnosis and management. However, common tear sampling methods present distinct limitation: Schirmer's strips frequently cause ocular irritation and discomfort due to their large contact area with the eye surface, while microcapillary tube collection could yield low reproducibility due to operator variability, especially when performed by different personnel. These limitations might compromise the accuracy and consistency of lipidomic data.

This study introduces a minimally invasive phenol red thread (PRT)-based sampling method optimized for tear lipidomics. The thin structure of PRT minimizes the risk of ocular irritation, allows rapid and gentle tear collection. This user-friendly and easy-to-perform method is suitable for subjects with reduced tear volume or lower tolerance for foreign body sensation, and it enables more reproducible sample collection by reducing operator-dependent variability.

Tear lipids collected by PRT were extracted using an optimized methanol/methyl tert-butyl ether (MTBE) phase separation protocol and analyzed by high-resolution LC-Orbitrap-IQX MS/MS with LipidSearch software. The workflow identified more than 700 unique tear lipid species and each

characterized by specific fatty-acid-derived product ions. These results indicate that PRT-based sampling provides robust lipid recovery for tear lipidomic analysis. This minimally invasive and reproducible approach offers a practical platform for clinical and experimental tear lipid research Ultimately, this could also facilitate biomarker discovery and disease monitoring.

INTRODUCTION:

Tear film is the outermost barrier that protects the ocular surface from pathogens and maintains ocular homeostasis [1,2], while lipid layer in tear film is the key component for preventing tear evaporation and maintaining tear film stability [3,4]. Dysregulation of the tear lipid composition has been linked to various eye diseases including dry eye disease (DED), meibomian gland dysfunction (MGD), and allergic conjunctivitis [5-8]. As a result, detailed characterization of tear lipidomics has become a critical and trending research direction for a more comprehensive understanding of the molecular pathologies underlying these ocular conditions [9].

While tear lipidomic approach holds significant clinical importance, a critical challenge in tear lipidomics is establishing a standardized, reproducible collection and analysis workflow. Current tear sampling methods, Schirmer's strips and microcapillary tube, each present distinct limitation. Schirmer's strips have a large contact area with the ocular surface that often cause ocular irritation and reflex tearing [9,10]. While microcapillary tube collection is highly dependent on operator technique and can yield inconsistent results when performed by different personnel [11]. Additionally, improper use of these sampling tools may also carry a risk of ocular surface injury. These limitation could compromise the accuracy and reproducibility of downstream data [12,13]. Furthermore, tear collection methods that require complex handling procedures may limit their accessibility and standardization across different laboratories and clinical settings. Therefore, there is a pressing need for establishing a non-invasive, robust, and reproducible tear collection and processing workflow that can preserve the native tear lipid profile.

Recent studies have highlighted the utility of phenol red thread (PRT) for downstream LC–MS/MS workflows analyses in proteomic and metabolomic applications [14,15]. PRT offers inherent advantages as a tear collection tool. Its thin structure, minimally invasive nature and straightforward sampling procedure could minimize technical complexity and reduce operator-dependent variability, while being well-tolerated by most subjects [14,16]. However, a standardized workflow specifically for PRT-based tear lipidomic analysis has not yet been established.

This protocol introduces an integrated workflow for reproducible tear lipidomics: a user-friendly PRT-based tear collection method adapted for lipid analysis, an optimized methyl tert-butyl ether (MTBE)/methanol lipid extraction protocol with defined solvent-to-sample ratios and storage controls [17,18], and a modified lipid identification method using high-resolution LC–MS/MS. This standardized approach enables a consistent tear lipidomic profiling for downstream biomarker discovery and disease characterization. Also, it is particularly well-suited for subjects with low tear volume or reduced tolerance for foreign body sensation. [**Figure 1**]

PROTOCOL:

Subjects provided written informed consent before participation in the study. The study was approved by the Institutional Review Board (IRB) of The Hong Kong Polytechnic University.

1. Phenol Red Thread (PRT)-Based Collection of Human Tear Fluid

1.1. Wear gloves and disinfect the workstation to prevent sample contamination.

1.2. Make sure the PRT package is sealed and not expired before opening it.

1.3. Hold the thread by the end opposite the bent hook. Always avoid touching the bent hook half of the PRT.

1.4. Ask the subject to look at the superior nasal direction.

1.5. Pull down the subject's lower eyelid gently and insert the PRT with the bent hook positioned in the lower temporal palpebral conjunctiva near the outer canthus of the subject's eye. [**Figure 2**]

1.6. Ask the subject to close the eyes gently and tilt his/her head slightly forward to prevent the PRT from touching his/her facial skin.

Note: Tear absorbed would gradually induce a red dye front indicating the sampled region of PRT due to the pH-sensitive phenol red. To ensure enough tear fluid for lipidomic analysis, collecting tear volume equivalent to 50 mm of PRT is preferred, typically completed within 2 min.

1.7. Pull down the subject's lower eyelid gently and pull out the sampled PRT. Avoid touching the sampled region.

1.8. Record the length of sampled region of the PRT and transfer the PRT into a new sample tube with the aid of sterile forceps.

Note: If the sampled PRT length <50 mm, kindly ask the subject to rest for 1-3 min and repeat the collection with the same eye. Transfer each sampled PRT to a separate sample tube.

1.9. Label and cap the sample tubes properly.

1.10. Clean the gloves and forceps thoroughly with methanol and clean paper wipe to remove any tear residuals or contaminants and then let dry.

1.11. Repeat Step 1.2. to 1.10. for the other eye.

1.12. Seal the sample tubes with parafilm and immediately store the sample at -20°C for temporary storage or -80°C for long-term storage. Avoid unnecessary freeze-and-thaw cycles and protect the sampled PRT from light.

2. Processing of Sampled PRT

2.1. Pre-wash the micro-scissors with methanol before processing the PRTs.

2.2. Discard the bent hook end (i.e., first 3 mm) of the PRTs. [**Figure 3**]

2.3. Cut the sampled region of the PRT (i.e., region above the phenol red dye front) into 2 mm pieces and transfer these pieces to a clean 1.5 mL organic solvent tolerable microcentrifuge tube.

3. MTBE/Methanol Biphasic Separation for Lipid Extraction from Sampled PRT

Safety warnings: MTBE and methanol are flammable and volatile. Use in a well-ventilated area or fume hood. Avoid open flames and sparks. Wear gloves and lab coat. Wash hands after handling.

3.1. Sit the samples on ice.

3.2. Add 232 µL of ice-cold methanol (MS grade) to each sample and vortex for 15 s.

3.3. Sonicate the samples in a pre-cooled ultrasonic cleanser for 15 min.

3.4. Add 774 µL of MTBE (HPLC grade) and 194 µL of deionized water to each sample and vortex for 15 s. The resultant volume ratio of MTBE:methanol:water in the sample should be 4:1.2:1 (v/v/v).

3.5. Sonicate the samples again in a pre-cooled ultrasonic cleanser for 15 min.

3.6. Incubate the sample in thermomixer for 8 hr at 4°C, with shaking at 1,200 rpm.

3.7. Let the mixture sit at room temperature for 10 min.

3.8. Centrifuge the resultant mixtures at 10,000 ×g for 10 min at 4°C.

3.9. After centrifugation, the mixtures are separated into two phases. [**Figure 4**]

Checkpoint: Upon completion of phase separation, inspect the sample for two distinct layers: the upper organic phase (MTBE-rich) should appear transparent with a slight milky opacity, while the lower aqueous phase exhibits a yellow coloration attributable to the

presence of phenol red indicator. Proceed only if these phase characteristics are observed, as shown in Figure 4.

3.10. Collect 700 µL of the upper organic fraction containing lipids carefully and transfer to a pre-chilled new 1.5 mL microcentrifuge tube.

3.11. Discard the lower aqueous phase containing residual MTBE and methanol as hazardous chemical waste. Collect waste in labeled, sealed containers and dispose of according to institutional hazardous waste protocols and local regulations. Do not pour solvents down the drain.

3.12. Dry the lipid extracts using a refrigerated SpeedVac concentrator for 4 hr at 4 °C.

Note: To ensure complete drying, periodically check the sample dryness starting at 2 hours. Extend the drying time as necessary until samples are fully dried.

3.13. Store the dried lipid extracts at -20 °C for short-term storage or -80 °C for long-term storage.

4. Reconstitution of Extracted Tear Lipid for LC–MS/MS Analysis

4.1. Reconstitute the dried lipid extracts with 50 µL of the ice-cold methanol (MS grade)/chloroform (HPLC grade) mix (1:1, v/v) as the solvent (1 µL of solvent per 1 mm of PRT used) and follow by sonication in a pre-cooled ultrasonic cleanser for 15 min.

4.2. Centrifuge the reconstituted lipid extracts at 14,000 ×g and 4 °C for 10 min.

4.3. Carefully transfer 40 µL of the supernatant to an autosampler glass vial with glass insert installed.

4.4. Tightly cap the vial with non-slit septum open top cap and ready for LC-MS/MS analysis.

5. Sample Acquisition by LC-MS/MS

5.1. For Liquid Chromatography, set the column chamber and sampler temperature at 50°C and 4°C respectively.

5.2. For each injection, load 5 µL of the sample onto reverse phase LC column (C18; 1.7 µm, 100 mm × 2.1 mm) and fractionate the lipids at a flow rate of 0.3 mL/min in a 24 min separation gradient. Use mobile phase A comprising a mixture of 60:40 acetonitrile (MS grade):water (v/v) with 5 mM ammonium formate (MS grade) and 0.1% (v/v) formic acid (MS grade) and mobile phase B containing 90:10 isopropanol (MS grade):acetonitrile (v/v) with 5 mM ammonium formate and 0.1% (v/v) formic acid. Use the following gradient: 0-2 min: 40% B; 2-2.5 min: 40% B; 2.5-3 min: 58% B;3-18 min: 99% B; 18-20 min: 99% B; 20-20.1 min: 40% B; 20.1-24 min: 40% B.

5.3. For Orbitrap Mass Spectrometer, set the spray voltage to +3.5 kV for positive mode or -2.5 kV for negative mode.

5.4. Adjust the sheath gas flow rate to 45 Arb and the auxiliary gas flow rate to 10 Arb.

5.5. Set the sweep gas flow rate to 2 Arb.

5.6. Set the ion transfer tube temperature to 300°C and the vaporizer temperature to 320°C.

5.7. Use data-dependent acquisition (DDA) mode.

5.8. For MS1, set the resolution to 120,000, scan range to 100-2000 m/z, standard AGC target, auto injection time, dynamic exclusion to 6 s and intensity threshold at 5.0e4.

5.9. For MS2, set the resolution to 15,000, auto scan range, standard AGC target, dynamic injection time, isolation window at 1.6 m/z, collision energy type as higher-energy collisional dissociation (HCD) and normalized collision energy (NCE) to stepped values of 15, 30, and 40.

5.10. Analyze raw data with LipidSearch software or other compatible platforms.

REPRESENTATIVE RESULTS:

Composite tear samples were collected from 16 healthy volunteers at three independent visits spaced two weeks apart (R1, oldest batch; R2, R3 collected sequentially two weeks after each). Using LipidSearch with stringent criteria (i.e., Signal-to-Noise Ratio ≥100, grade C or above and ion intensity ≥30,000), we identified 773, 890, and 1,025 unique lipid species in R1-R3 respectively (Figure 5A). Positive ion mode identified 1,302 lipid species with 26.0% Grade A (i.e., both lipid class and all fatty acid chains belonging to a given lipid were completely identified; 338 lipids), 13.2% Grade B (i.e., full identification of lipid class and partial identification of fatty acid chains), and 60.8% Grade C (i.e., lipid class specific ion or fatty-acid-derived product ions were detected). Negative ion mode identified 257 unique lipid species with 8.2% Grade A (21 lipids), 22.9% Grade B, and 68.9% Grade C (Figure 5B). Combined analysis detected 1,559 total unique lipid species across both modes. Despite storage-related variations, 394 species consistently identified across all three sample sets (Figure 6). Intra-subject coefficient of variation across the three visits was 21.5% (positive mode) and 31.3% (negative mode), with 23 lipid classes recovered across both ionization modes (Figures 7A–B).

Compared to prior tear lipidome studies [4,19], , this workflow detected 1,559 unique species with substantially improved structural characterization. The expanded coverage reflects high-resolution LC-MS/MS separation, stringent identification criteria minimizing artifacts, and complementary dual-mode analysis. Notably, unlike prior studies lacking identification confidence metrics, this workflow provides transparent Grade-level reporting: positive ion mode yielded 338 Grade A identifications (highest structural confidence), while negative ion mode

contributed complementary lipid diversity. The consistent core lipid set (394 species) across different storage durations, combined with robust positive mode Grade A representation (26%) and acceptable intra-subject CVs, demonstrates reliable detection suitable for quantitative tear lipidomic studies.

FIGURE, TABLE AND LEGENDS:

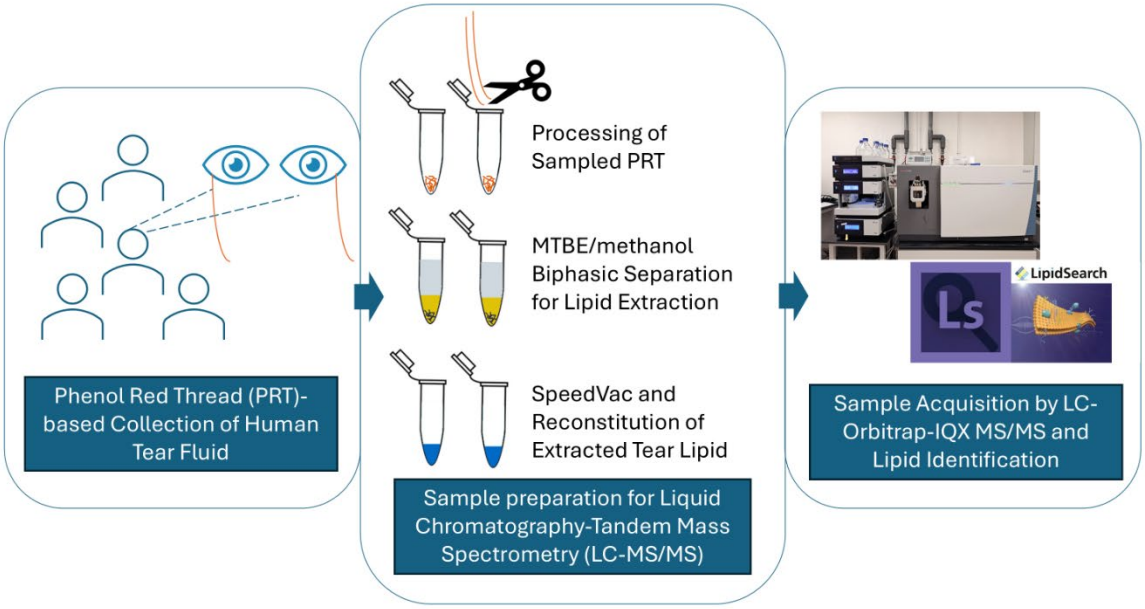

Figure 1: Schematic overview of the Phenol Red Thread (PRT)-based workflow for human tear lipidomics. Tear fluid is collected from subjects utilizing the PRT approach, which enables consistent sample acquisition with minimal discomfort. Sample processing involves sequential MTBE/methanol biphasic extraction, SpeedVac-mediated concentration, and reconstitution of tear lipid extracts for liquid chromatography-tandem mass spectrometry (LC–MS/MS). Lipidomic profiles are subsequently acquired via LC–MS/MS and subjected to annotation and identification using LipidSearch software.

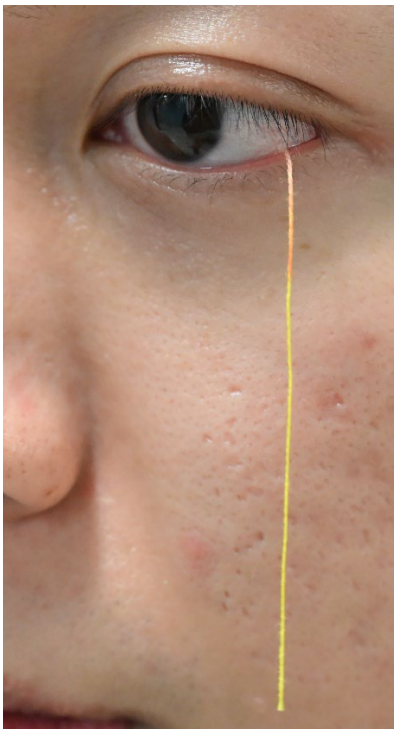

Figure 2: Position of the phenol red thread during tear collection.

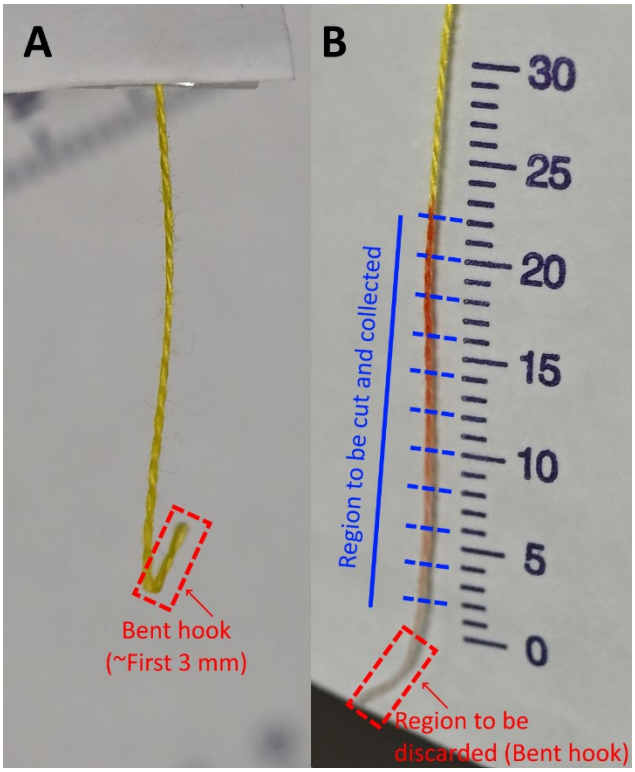

Figure 3: Physical appearance of the phenol red thread (PRT): (A) Unused PRT with bent hook end, (B) Used PRT with indicated section cut and collect for lipid extraction.

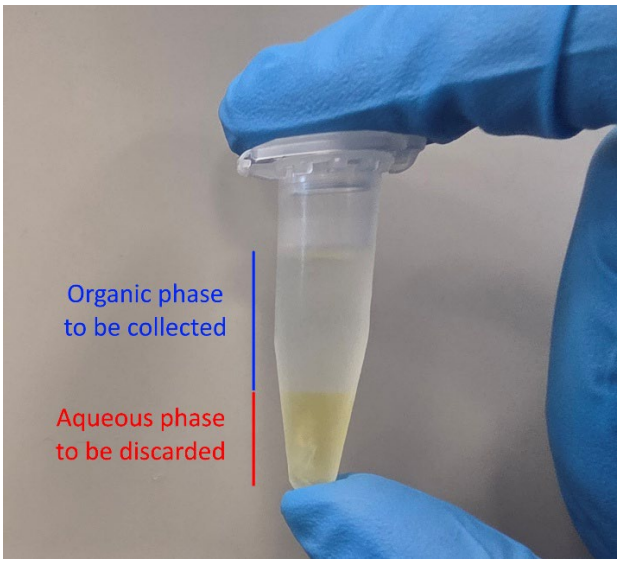

Figure 4: Phase separation of lipid extraction using MTBE-methanol-water: upper organic phase and lower aqueous phase.

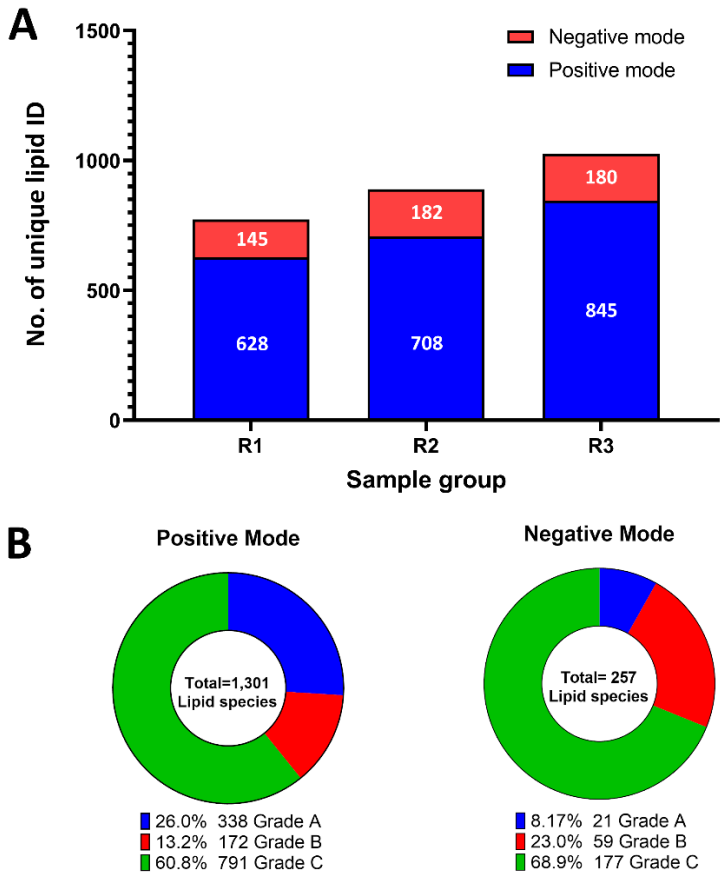

Figure 5: Lipid identification summary and quality assessment. (A) Total unique lipid species identified in each sample group (R1, R2, R3) by ionization mode. (B) Grade-level confidence distribution of lipid identifications in positive and negative ion modes.

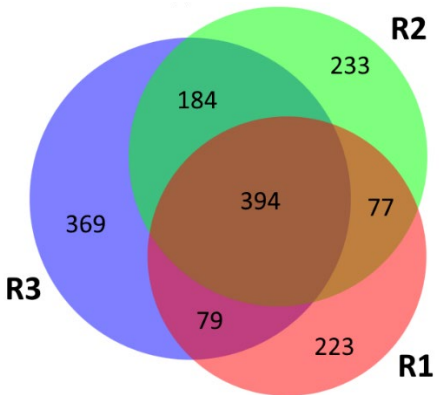

Figure 6: Venn diagram showing overlapping unique lipid species identified across three sample groups. Each circle represents the union of unique lipid species found in one sample group, while the intersections show lipid species that were commonly found in two or all three groups. This visualization highlights consistent and distinct lipid species among the groups.

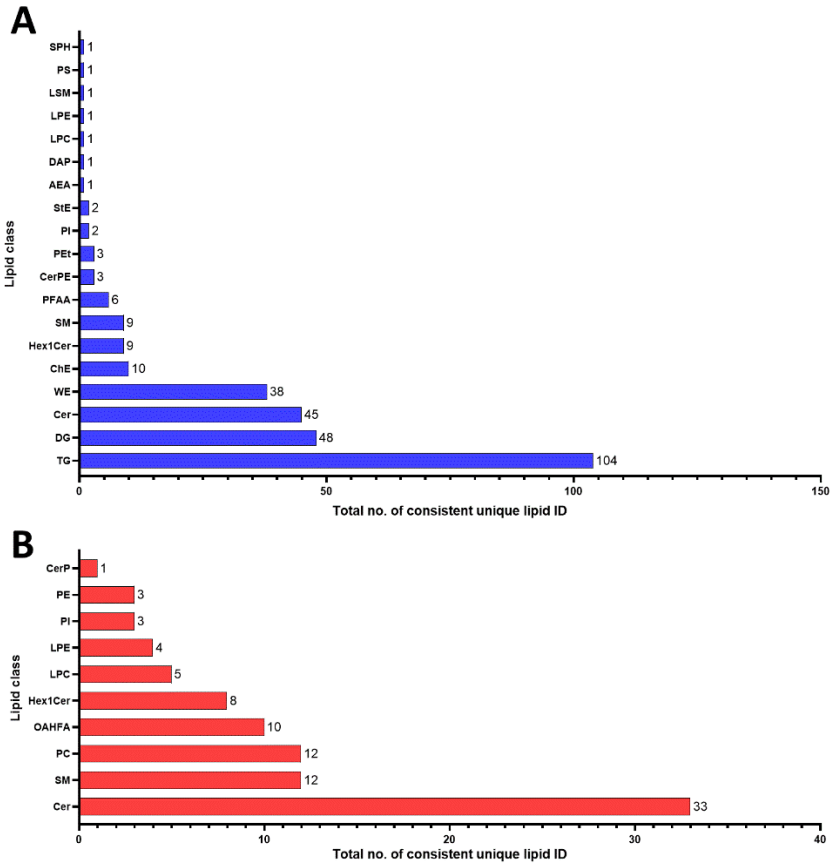

Figure 7: Consistent lipid class distribution across sample replicates. Distribution of unique lipid species identified within each lipid class by LC-MS/MS in (A) positive ion mode and (B) negative ion mode. Bars represent the number of unique lipids consistently identified across all three sample sets (R1–R3). Major lipid classes shown include: glycerolipids (TG, DG), sphingolipids (Cer, SM, Hex1Cer), phospholipids (PC, PE, PI, PS, PEt), lysophospholipids (LPC, LPE), and other minor species.

DISCUSSION:

Advantages of PRT-Based Tear Sampling and Lipid Extraction

The PRT-based tear lipidomics workflow presented here provides a minimally invasive and practical sampling method for comprehensive tear lipid profiling. Compared to Schirmer strips, which require prolonged contact and larger sample volumes, PRT sampling is rapid that can be completed within 2 minutes, well-tolerated by diverse populations including those with reduced tear volume or heightened ocular sensitivity, and yields tear samples compatible with high-resolution LC-MS lipidomics [14,20]. Unlike microcapillary tube collection, which is time-consuming and requires precise technical handling and operator expertise, PRT-based sampling requires minimal training and can be readily performed in clinical settings with high reproducibility and ease of use [14]. Regarding the lipid extraction method, MTBE/methanol-based phase separation avoids chloroform toxicity while providing comparable or superior lipid recovery to traditional Folch and Bligh-Dyer methods for most lipid classes, though it may show slightly reduced recovery for certain polar lysophospholipids (LPC, LPE). This workflow successfully identified over 700 unique tear lipid species across 23 lipid classes, substantially expanding previous tear lipidome datasets [4,19].

Technical Considerations and troubleshooting

Several factors are critical for reproducible results. The reagent volumes specified in this protocol (232 µL methanol, 774 µL MTBE, 194 µL water for lipid extraction; 50 µL methanol/chloroform for reconstitution) are optimized for a standard 50 mm PRT sample, yielding a MTBE/methanol/water ratio of approximately 4:1.2:1 (v/v/v). This ratio was selected based on optimization studies demonstrating robust extraction efficiency and reproducibility across diverse sample matrices [18,21]. Sample-to-solvent ratio is a critical parameter that directly influences lipid yield and analytical sensitivity. For samples with collected PRT lengths different from 50 mm, users must adjust solvent volumes proportionally according to the per-mm PRT-to-solvent ratios where lipid extraction requires 24 µL MTBE-methanol-water mix per 1 mm of PRT, and reconstitution requires 1 µL chloroform-methanol mix per 1 mm.

Strict adherence to this solvent ratio is critical for optimal phase separation and signal intensity. If insufficient PRT is collected (e.g., <50 mm) without reducing reconstitution solvent volumes proportionally, the resulting low sample concentration yields diminished MS signal intensity, readily masked by background noise, substantially reducing the number of identifiable lipid species and spectral quality. The reduced solute concentration diminishes ionization efficiency and detection capacity in electrospray ionization-based MS workflows. Conversely, if PRT exceeds the standard amount (e.g., >50 mm) without proportionally increasing solvent volumes, excessive sample material absorbs and retains water, reducing the aqueous phase capacity to solubilize hydrophilic components. This inefficient phase separation causes the lower aqueous phase to turn red instead of the expected yellow, indicating incomplete partitioning. Phenol red and other hydrophilic contaminants then migrate into the upper organic phase. Upon drying, such samples display deep red or brownish yellow coloration—a visual indicator of phenol red contamination and ion suppression risk in subsequent MS analysis. If this coloration occurs, redissolve the sample and repeat the lipid extraction steps 3.2-3.12 to achieve clean phase

separation and remove interfering substances. Careful documentation of PRT length for each sample is essential to maintain analytical consistency [22,23]. A gradual loss of detectable lipid species was observed in older samples (R1). This highlighted the importance of immediate transfer of sampled PRT to a dark environment at -80°C for preventing enzymatic and oxidative degradation [9,24,25]. This also underscored the importance of standardized storage protocols for large cohort studies.

Limitations

This workflow has several notable limitations. The requirement for participants to maintain upward gaze during PRT insertion may be challenging for individuals with nystagmus or severe ocular surface discomfort. Additionally, the workflow has been optimized for high-resolution Orbitrap MS/MS platforms and may require additional validation for other MS platforms. Besides, previous studies investigated that the MTBE extraction method might exhibit reduced recovery for certain polar lipid classes, particularly lysophospholipids (LPC, LPE), compared to chloroform-based approaches such as Folch and Bligh-Dyer methods [26]. Despite these limitations, the PRT-based approach remains a practical and minimally invasive alternative for tear biomarker discovery in ophthalmology and systemic disease research.

Future applications and clinical significance

The enhanced lipid coverage provided by this workflow supports multiple translational applications. In the near future, tear lipid profiling can identify and validate biomarkers for ocular diseases such as dry eye disease and meibomian gland dysfunction or other systemic conditions associated with tear lipid alterations [27]. The rapid sampling and standardized processing enable longitudinal studies to monitor disease progression and treatment responses. Long-term perspectives include integration with proteomics and metabolomics for multi-omics profiling, this would enable personalized medicine approaches and point-of-care diagnostics [12,28,29]. Given the minimally invasive nature and robust lipid identification capabilities, PRT-based tear lipidomics is well-positioned for large-scale population screening and precision ophthalmological applications.

ACKNOWLEDGMENTS:

This work was supported by the InnoHK initiative and the Hong Kong Special Administrative Region Government and Research Centre for SHARP Vision at The Hong Kong Polytechnic University. The authors also gratefully acknowledge technical support from the University Research Facility in Chemical and Environmental Analysis (UCEA) and the University Research Facility in Life Sciences (ULS) of The Hong Kong Polytechnic University.

DISCLOSURES:

The authors have no conflicts of interest to declare.

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