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# A simple and reproducible method for quantification of human tear lipids with ultrahigh-performance liquid chromatography-mass spectrometry

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**Purpose:** The purpose was to select a simple and reproducible method for lipid measurements of human tears with ultrahigh-performance liquid chromatography-mass spectrometry (UHPLC-MS). Two sample preparation procedures were evaluated and compared: the Bligh and Dyer (BD) liquid-liquid extraction method with chloroform and methanol and protein precipitation with isopropanol (IPA).

**Methods:** Reproducibility and recovery efficiencies of 20 non-endogenous internal lipid standards were tested in 10-µl tear samples from healthy subjects. The lipid coverage and the simplicity of execution were also assessed. Lipid profiles of the tear extracts were acquired with UHPLC-MS, uhpland the lipids were identified using SimLipid software.

**Results:** Both methods were robust producing good lipid coverage and reproducibility and high recovery efficiencies. The two protocols identified a 69-feature tear lipidome that covered 11 lipid classes from six different lipid categories. The main differences in recovery were due to the intrinsic lipid selectivity of each solvent. Although both methods were similarly efficient in recovering *O*-acyl-ω-hydroxy fatty acid (OAHFAs) and non-polar lipids, polar lipids were more efficiently recovered with IPA precipitation, which, in turn, exhibited higher reproducibility. In addition, IPA precipitation is automatable and simpler than the BD approach.

**Conclusions:** IPA precipitation is an excellent procedure for extracting lipids from small tear volumes for quantitative large-scale, untargeted lipid profiling, which may be useful for identifying lipid biomarkers in tears from patients with different ocular surface pathologies, allowing personalized therapies to be designed.

The tear film covers the anterior surface of the cornea and helps maintain the homeostasis of the ocular surface [1]. This film is the first refractive interface for incident light, and thus, the tear film plays an important role in ensuring good vision [2]. Tears hydrate and lubricate the mucous membranes that constitute the ocular surface, supplying nourishment to the avascular corneal epithelium and providing a smooth optical surface that is essential for visual acuity. The drainage of tears also represents the first line of defense for the anterior eye against invading pathogens. The tear film is an exceptionally complex structure, composed of water, inorganic salts, carbohydrates, lipids, and proteins [3]. The precise physiologic balance of these various metabolites is crucial in ensuring its correct function and maintaining its biophysical integrity. Perturbations in this delicate equilibrium may manifest as distinct ocular conditions, such as dry eye syndrome (DES) and meibomian gland dysfunction (MGD) [4,5].

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The lipid composition of human meibomian gland secretions, the predominant source of lipids in precorneal tear film, has been elucidated in considerable detail, benefiting from the advances in mass spectrometry (MS) [6-12]. Comprehensive lipidomic analyses of tear fluids will be imperative to elucidate any changes in the lipid composition of the tear film that might alter its biophysical properties in the case of disease, which will help guide the appropriate treatment for each patient. The precorneal tear film behaves as a single dynamic functional unit with different compartments [13]. The external lipid layer mainly contains non-polar lipids, while the internal stratum is made up of polar lipids [14]. Additional insight into the nature of these layers was obtained from the multilamellar sandwich model of the tear film lipid layer [15]. The non-polar lipids in the external layer have been hypothesized to prevent tear evaporation, to provide a clear optical surface, and to present a barrier against foreign objects and organisms [16]. In contrast, the amphiphilic

properties of the polar lipids allow them to form an interface between the external lipid layer and the mucus or aqueous layer. This interface may render structural stability by lowering the surface tension of the aqueous tears, increasing viscoelasticity, and promoting the correct segregation of the tear film molecules, allowing the normal spreading of tears and preventing ocular surface dewetting [17,18].

Until recently, the challenge of elucidating the components of tears has been to overcome the limitations imposed by the analytical and biochemical techniques available. The sensitivity and resolution of most analytical techniques require large samples, leading to the pooling of samples, or chemical derivatization for detection, or both methods. However, several analytical techniques are now available for detecting and quantifying lipids in tear samples, in particular, MS coupled with liquid chromatography (LC) separation [19]. Lipidomic research is undergoing remarkable developments, but standard protocols are still needed. A critical step in any lipidomic study is the extraction of lipids from their biologic matrix, as sample preparation can have a strong impact on the original sample composition. The most universal extraction methods for obtaining lipids from biologic fluids are the biphasic chloroform-based extraction techniques described by Folch and colleagues [20] and Bligh and Dyer (BD) [21]. These two methods are based on the separation of lipids from polar metabolites via their partition in defined proportions of chloroform, methanol, and water. Another strategy that can now aid lipid sample preparation is monophasic organic solvent-based protein precipitation. Considering the extractability of lipids and the efficiency, repeatability, and simplicity of protein removal, Sarafian and colleagues found that protein precipitation with isopropanol (IPA) was the most suitable preparative method for analyzing lipids in plasma [22]. The lipidomic studies performed to date on tears have mainly used chloroform and methanol [23] or tert-butyl methyl ether:methanol to extract the lipids from tear pools [12]. However, to the best of our knowledge, there are no studies comparing different extraction systems to quantitatively analyze the lipids in tears.

Given that the maximum volumes usually available to study tear samples from individual patients normally limit such analyses, a study comparing biphasic and monophasic methods is needed. Accordingly, the aim of the present study was to evaluate and compare two sample preparation protocols for tear lipid analysis with ultrahigh-performance liquid chromatography (UHPLC)-MS. One is the universal chloroform:methanol-based BD method, and the other is a protein precipitation method using IPA. Thus, the goal of this study is to identify which of these two lipid extraction methods is the most robust and efficient for use in the quantification of tear lipids.

## METHODS

*Study design:* This study was performed in two steps, an extraction step and an identification phase (as detailed in Figure 1). In the extraction step, tear samples were collected, pooled, and then immediately separated into two sets of ten replicates (10 µl each). For each method of lipid recovery, a mix of internal standards (ISs) that contained all lipid classes (Table 1) was added to five aliquots before (prespiked) and five after (post-spiked) extraction. This enabled us to compare the recovery efficiency, reproducibility, and lipid coverage of the two sample preparation protocols in the second step. In the identification phase, the tear lipidome was analyzed with UHPLC-MS to identify and quantify the lipid species present in normal tears. For this purpose, a blank, consisting of 90:10 (v/v) methanol:toluene solution, which was the chosen resuspension solvent, was included at the beginning and the end of the run to test any possible contamination or carryover effect. A quality control (QC) sample, prepared by combining equal aliquots (10 µl) of the replicates from each sample preparation method, was injected regularly every five injections throughout the run to monitor the sensitivity and the stability of the UHPLC-electrospray ionization-quadrupole time of flight (UHPLC-Electrospray ionization-quadrupole time of flight-ESI-QTOF) platform. This QC sample was also used to condition the system at the beginning of the analysis. It was observed in a previous test that at least 15 injections of a sample containing the matrix studied were necessary to stabilize the system. The order of injection of the samples (replicates of each protocol) was randomized to minimize the effect of the instrumental drift arising from column degradation or contamination of the MS source on the evaluation of reproducibility within each protocol.

*Tear fluid collection:* Medically qualified personnel conducted this study after approval was received from the Cruces Hospital Ethics Committee, and it was performed in accordance with the tenets of the Declaration of Helsinki on Biomedical Research and adhered to the ARVO statement involving Human Subjects. Before tear collection, written informed consent was obtained from all subjects, once the nature and possible consequences of the study had been explained. The inclusion criteria were based on clinical examination including the Schirmer I test with anesthesia to measure the basal secretion, slit-lamp examination of the lid margin and meibomian glands, fluorescein staining results according to the Oxford scale, tear film break-up time (TFBUT), and tear osmolarity. The following exclusion

criteria were applied: ocular surgery performed within the preceding 3 months, a systemic condition (active allergy or DES) or the use of medication (anti-inflammatory agents) that could interfere with the interpretation of the results, or the concomitant administration of topical medication (except artificial tears).

*Subjects:* Ten healthy volunteers were enrolled at the Cornea Unit of the Instituto Clínico-Quirurgico de Oftalmología (ICQO), Bilbao, Spain. Six women and four men (age 27±2.5 years) were included in the study. Ten healthy volunteers were enrolled in the study (age 27±2.5 years, six women and four men). They presented corneal staining with fluorescein (grade 0), Schirmer I test (15±5.8 mm/5 min), tear osmolarity (296±2.58 mOsm/ml), meibomian gland analysis (grade 0), and TBUT (12.0±1.03 s).

*Sample collection:* Tear samples were collected from the lower conjunctival sac using 10-µl glass capillaries, as described previously [24]. The collection was performed in multiple sessions until the volume was obtained avoiding irritation of the conjunctiva to minimize cell contamination and sample dilution as a result of reflex tearing. The samples were stored at −80 °C until extraction.

*Chemicals and standards:* Optima® LC/MS-grade water; methanol, acetonitrile, 2-propanol, and formic acid were obtained from Fisher Scientific (Fair Lawn, NJ). HPLC-grade chloroform, leucine enkephalin acetate hydrate, ammonium

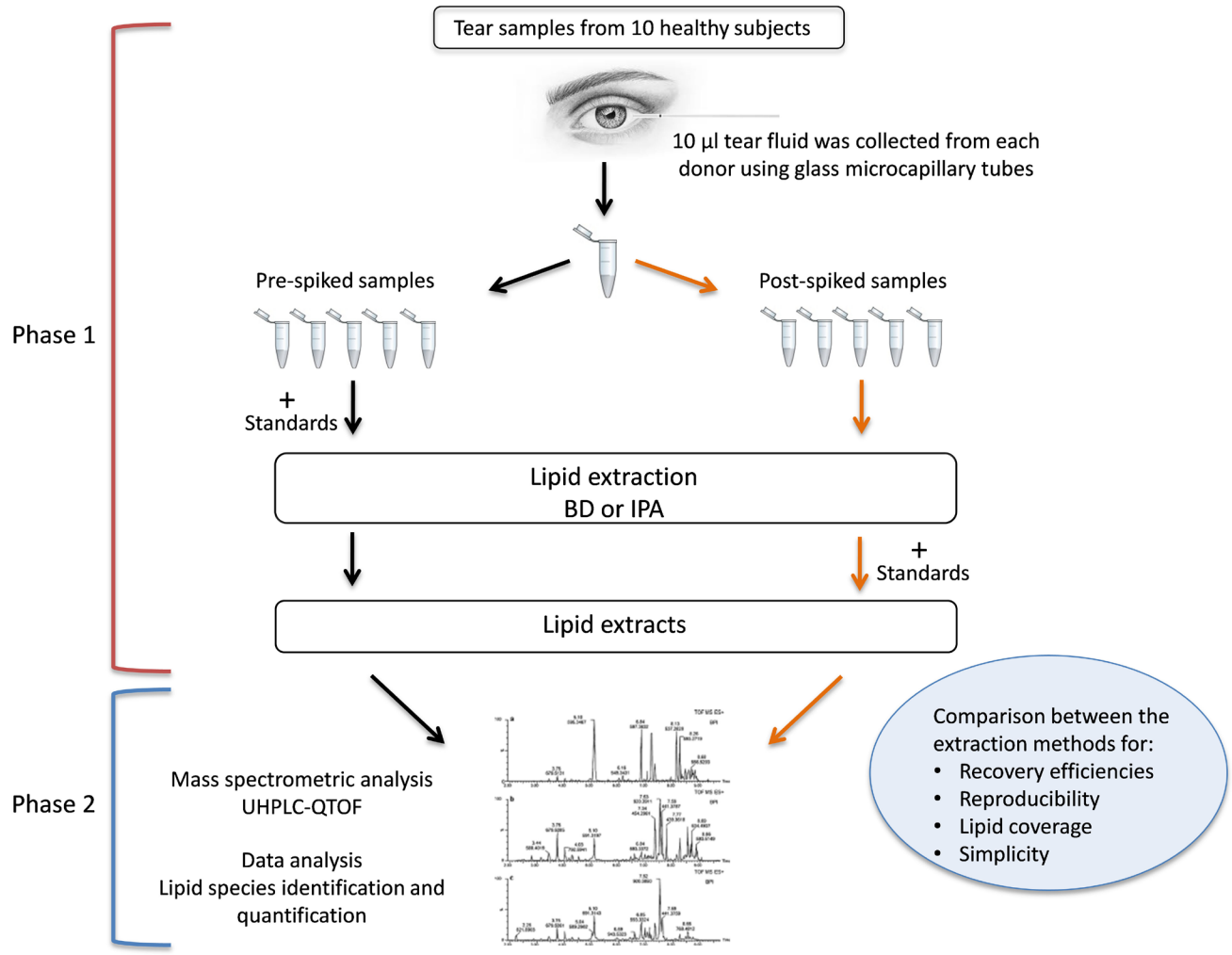


Figure 1. Workflow for untargeted lipid profiling with UHPLC-QTOF of tears using the two lipid extraction methods under comparison. Tear samples were collected individually from ten healthy subjects. A pool was made to evaluate in all cases the same sample and divided into five replicates of 10 µl to be extracted with the internal standard (IS) added before the extraction (prespiked) and another five replicates of 10 µl each with the IS added after the lipid extraction of the tear (post-spiked). The extracts were analyzed with ultrahigh-performance liquid chromatography-quadrupole time of flight (UHPLC-QTOF), and the lipid species obtained were identified and quantified.

**Table 1. Linearity and sensitivity values of the quantified internal standards.**

| Comp. No | Lipid | Linear range (ug/ml) | Slope | OO | $R^2$ | RSD % (n=3) | LOD (µg/ml) | LOQ (µg/ml) |
|---|---|---|---|---|---|---|---|---|
| 3 | 18:1(d7) Lyso PC | 0.05–2.55 | 795 | −47 | 0.9992 | 0.04 | 0.062 | 0.068 |
| | | 2.55–25.50 | 3161 | −8299 | 0.9954 | 0.4 | | |
| 5 | Lactosyl(β) C12 Ceramide | 0.2–21 | 54 | −28 | 0.9972 | 0.2 | 0.19 | 0.24 |
| 6 | Glucosyl(β) C12 Ceramide | 0.03–1.61 | 358 | −8 | 0.9995 | 0.03 | 0.033 | 0.046 |
| | | 1.61–16.09 | 567 | −450 | 0.9994 | 0.08 | | |
| 7 | Ceramide (C12) | 0.03–12.04 | 1323 | −51 | 0.9972 | 0.3 | 0.024 | 0.03 |
| 8 | d18:1–18:1(d9) SM | 0.074–10.34 | 583 | −522 | 0.9759 | 1 | 0.065 | 0.074 |
| 9 | 15:0–18:1(d7) PC | 0.32–16.07 | 348 | −193 | 0.9982 | 0.02 | 0.17 | 0.26 |
| | | 16.07–160.7 | 1972 | −36084 | 0.993 | 0.5 | | |
| 10 | 15:0–18:1(d7) DG | 0.094–9.4 | 298 | −83 | 0.9977 | 0.2 | 0.064 | 0.078 |
| 11 | Ceramide (C25) | 0.05–1.67 | 442 | −5 | 0.9998 | 0.01 | 0.038 | 0.048 |
| 12 | 15:0–18:1(d7) −15:0 TG | 0.1–5.7 | 795 | −150 | 0.9983 | 0.03 | 0.15 | 0.15 |
| | | 5.7–57.3 | 3241 | −19370 | 0.9937 | 0.6 | | |
| 13 | 18:1(d7) Chol Ester | 0.7–356.1 | 1.2 | 219.5 | 0.9947 | 0.02 | 0.47 | 2.82 |
| 14 | 18:1(d9)-26:0 WE | 1.2–120 | 2 | 2 | 0.9998 | 0.02 | 0.29 | 1.26 |
| 15 | 18:1(d7) Lyso PE | 0.53–5.30 | 60 | −10 | 0.9998 | 0.03 | 0.23 | 0.28 |
| 16 | 15:0–18:1(d7) PI (NH4 Salt) | 0.91–9.10 | 67 | −26 | 0.9995 | 0.04 | 0.3 | 0.38 |
| 17 | 15:0–18:1(d7) PS (Na Salt) | 1.4–4.2 | 3 | −0.1 | 0.9952 | 0.09 | 0.42 | 1.4 |
| 18 | 15:0–18:1(d7) PG (Na Salt) | 0.29–29.1 | 47 | −46 | 0.9956 | 0.3 | 0.30 | 0.38 |
| 19 | 15:0–18:1(d7) PE | 0.57–5.70 | 46 | −7 | 0.998 | 0.2 | 0.14 | 0.22 |
| 20 | 18:1–16:0 OAHFA | 0.2–40 | 37 | −34 | 0.9955 | 0.4 | 0.1 | 0.27 |

Abbreviations: LOD, limit of detection; LOQ, limit of quantification

formate, and sodium hydroxide solution were purchased from Sigma-Aldrich (Sigma Chemical Co., St Louis, MO). The Splash™ LipidoMix™ and the ceramide:sphingoid internal standard mixtures (Appendix 1) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). In-house synthesis of the *O*-acyl-ω-hydroxy fatty acid (OAHFA) standard, 16-(oleoyloxy) hexadecanoic acid, was carried out by the procedure already reported in the literature [25]. The wax ester (WE) standard—15,15,16,16,17,17,18,18,18-d9 (WE 18:1(d9)/26:0)—was obtained by treating deuterated oleic acid with $SOCl_2$ [26]. Both compounds were characterized with $^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR) spectroscopyand with high-resolution mass spectrometry, and their purity was established as >95%. The crude products were purified with column chromatography (Appendix 2).

*Lipid preparation:* Lipids were extracted exhaustively from tear fluid with the BD biphasic method [21] using glass tubes. Briefly, 10 µl of tear fluid was diluted with 90 µl of ultrapure water to obtain a volume that was easy to handle, and then, each mix of ISs (10 µl, Appendix 1) was added to the prespiked group. The samples were then mixed thoroughly with 15 volumes of chloroform:methanol (1:2, v:v) and subsequently, with 5 volumes of chloroform and 24 volumes of ultrapure water. After centrifugation at 1,100 ×*g* (4 °C, 15 min), the lower layer (organic phase) was transferred to a new tube, and the methanol phase and the protein pellet were reextracted with 36 volumes of chloroform:methanol:water (1:1:1, v:v:v). The tubes were then centrifuged as before, the two chloroform phases were combined, the chloroform was evaporated (Thermo Scientific Savant A-290), and the dried extracts were stored at −80 °C under an atmosphere of nitrogen. An identical lipid extraction procedure was used for the post-spiked group, except that the ISs were added to samples once the lipids had been extracted.

*IPA precipitation:* This method is an adaptation of the monophasic procedure described previously [22]. Ten replicates of pooled tear fluid (10 µl) were prepared in Eppendorf tubes. The replicates were diluted with 90 µl of ultrapure water, and the ISs were added as above. Four volumes of precooled isopropanol (−20 °C) were added to the samples to precipitate the protein; the samples were mixed thoroughly, incubated at room temperature for 10 min, and then centrifuged at 14,000 ×*g* (4 °C, 20 min). The upper 80% of the supernatant volume was collected to avoid contamination with the components of the pellet, the isopropanol was evaporated, and the dried extracts were stored at −80 °C under nitrogen.

*UHPLC-MS analysis:* UHPLC was performed using an ACQUITY UPLC™ system from Waters (Milford, MA), equipped with a binary solvent delivery pump, an autosampler, and a column oven. A reverse-phase column (ACQUITY UPLC $C_{18}$ CSH, 100 × 2.1 mm, 1.7 µm) and a precolumn (ACQUITY UPLC $C_{18}$ CSH 1.7 µm VanGuardT™) were used at 65 °C to separate individual lipids. The mobile phases consisted of acetonitrile and water (40:60, v/v) with 10 mM ammonium formate and 0.1% formic acid (phase A), and acetonitrile and isopropanol (10:90, v/v) with 10 mM ammonium formate and 0.1% formic acid (phase B). The flow rate was 500 µl/min, and the injection volume was 7.5 µl. All samples were kept at 4 °C before the analysis. (Appendix 3 shows the gradient used for the lipid profiling by UHPLC-MS).

All UHPLC-MS data were acquired on a SYNAPT G2 HDMS, with a QTOF configuration (Waters) equipped with an ESI source that can be operated in positive (+) and negative (-) modes. The capillary voltage was set to 1 kV (ESI+ and ESI−). Nitrogen was used as the desolvation and cone gas, at flow rates of 1,000 l/h and 10 l/h, respectively. The source temperature was 120 °C, and the desolvation temperature was 500 °C.

Leucine-enkephalin solution (2 ng/µl) in acetonitrile:water (50:50, v/v) with 0.1% formic acid was used for lock-mass correction. The ions at mass-to-charge ratios (m/z) 556.2771 and 278.1141, or 554.2615 and 236.1035, depending on the ionization mode from this solution, were monitored at scan time 0.3 s, and at 10-s intervals, three scans, on average, using a mass window of ±0.5 Da. Other conditions were lock spray capillary of 2.0 and 2.5 kV and collision energy of 21 and 30 eV in ESI+ or ESI–, respectively. The reference internal standard was introduced into the lock mass sprayer at a constant flow rate of 10 µl/min, using an external pump.

All acquired spectra were automatically corrected during acquisition using the lock mass. Before analysis, the mass spectrometer was calibrated with a 0.5 mM sodium formate solution.

Data acquisition took place over the mass range 50–1,200 u in resolution mode (full width at half maximum, FWHM≈20,000) with a scan time of 0.3 s and an inter-scan delay of 0.024 s. The cone voltage was set to 40 V (ESI+ and ESI–). The mass spectrometer was operated in the continuum MSE acquisition mode for both polarities. During this acquisition method, the first quadrupole Q1 was operated in a wide-band rf-only mode, allowing all ions to enter the T-wave collision cell. Two discrete and independent interleaved acquisition functions were automatically created: The first function, typically set at 6 eV, collected low-energy or unfragmented data, whereas the second function collected high-energy or fragmented data, typically obtained by using

a collision energy ramp from 15 to 40 eV. In both cases, argon gas was used for collision-induced dissociation. In this way, the first function (low energy) was used to assign the precursor ion, whereas the second function (high energy) was employed to obtain different characteristic fragment ions of the lipids.

*MS data processing:* Data were acquired as raw files with the MassLynx V4.1 software, converted into NetCDF files using the DataBridge 3.5 converter (Waters), and grouped according to the different sample preparation protocols. The blanks and the QC samples were treated as separate groups. The NetCDF files were processed using XCMS 1.42.0 software (Metlin, La Jolla, CA) to convert the three-dimensional LC-MS data into a table of time-aligned features, using the retention time (RT), *m/z*, and intensity for each sample. XCMS was written in the R statistical programming language (v 3.2.2.), which is freely available under an open-source license. The matched filter detection algorithm was used for peak identification, and to avoid LC-MS artifacts, and the CAMERA package was used to filter the peaks detected. The peak marker tables generated (comprising *m/z*-RT pairs and their corresponding intensities for each sample) were exported into SPSS to carry out a univariate analysis, and into SIMCA 14.1 (Umetrics, Umea, Sweden) for the multivariate analysis.

*Assessment of recovery:* The BD and IPA methods were evaluated by spiking 10 µl of each IS mixture into the sample before and after lipid extraction was performed. A previous analysis of the ISs with UHPLC-MS with ESI in the positive (+) and negative (-) modes (ESI+ and ESI–) allowed the *m/z* ratios to be characterized, as well as the retention times for the molecular ion adducts and fragments. The most intense ion peak was selected for the recovery calculations for each standard (Appendix 4), and the recovery of each IS lipid was then determined as the ratio of the peak area measured in the samples spiked before and after the extraction procedure.

*Reproducibility:* The reproducibility of each method was assessed by analyzing the lipids in ten replicates (pre- and post-spiked), and the data were processed using univariate and multivariate statistics. Other parameters were calculated directly from the resulting XCMS table.

*Statistical analyses:* Univariate statistics were used to compare the coefficient of variation (CV) distribution of the feature intensity among the replicates prepared with each protocol. A histogram of the CV values was plotted for each sample preparation method to compare the CV distribution between the IPA and BD protocols. The CV distribution was also compared by calculating the percentage (or number) of features that had a CV lower than 20%, an acceptable value in biomarker analysis. In addition, instrumental variability was estimated by computing the CV in the QC samples, obtained by pooling 20 µl of each of the 20 extracts. The CV of the IPA, BD, and QC groups was compared using a non-parametric Kruskal–Wallis analysis of variance (ANOVA). All statistical analyses were performed using SPSS version 20, and a p value of less than 0.05 was considered statistically significant.

*Multivariate statistical analysis:* The repeatability of the sample preparation methods was also assessed using a principal component analysis (PCA). The PCA was performed using SIMCA 14.1 (Umetrics). The data sets were logarithm-transformed, 10log (peak area), and mean centered. The score plot was examined to assess the degree of similarity between the different protocols, and the discriminating features between lipidic profiles were identified for each model by displaying loading plots (model coefficients versus covariance).

*Linearity and sensitivity:* To evaluate the linearity of the method, samples (10 µl) were prespiked with the ISs at five different final concentrations, ranging from 0.030 to 356.1 µg/ml (n = 3), and calibration curves were constructed for the IS lipids. The $R^2$ linear regression coefficients, relative standard deviation (RSD) values, slopes, and intercepts were calculated. Thus, the sensitivity of the method was studied by determining the calibration curve slopes, and the limit of detection (LOD) and quantification (LOQ) values were calculated according to the International Union of Pure and Applied Chemistry (IUPAC) method:

$$LOD=3(S/N)$$

$$LOQ=10(S/N)$$

where (S/N) corresponds to the signal-to-noise ratio obtained with the MassLynx software.

*Lipid identification and quantification:* The data generated with UHPLC-MS were extracted using $MS^E$ Data Viewer (Waters MS Technologies, Manchester, UK), software that was also used to align the precursor and product spectra according to their retention times, generating an exportable text file that could be used for lipid identification with the SimLipid software (Premier Biosoft International, Palo Alto, CA). The molecular weight of the compounds was defined using the low-collision energy $MS^E$ spectrum in positive and negative modes, while the fragmentation yielded by the high-collision energy $MS^E$ spectrum was used to elucidate other structural details. Tolerance for MS and MS-MS identification was set at 5 mDa.

Quantification was performed using XCMS-extracted intensities of features previously identified as individual lipid species. Quantification was conducted via normalization of

the intensity of the monoisotopic peak of each native species to the intensity of the monoisotopic peak of internal standard. The internal standards used in this study were chosen not to be natively present in human tears.

## RESULTS

*Lipid coverage:* The extracted ion chromatograms from the tear samples spiked with the ISs were examined in positive (Figure 2A) and negative (Figure 2B) ESI modes. From the features detected with the full XCMS platform (4,232 in ESI+ and 382 in ESI–), 69 were considered (signal-to-noise ratio >3) and identified as individual lipid species in tear samples. These species corresponded to 11 lipid classes of six different lipid categories: fatty acids (FAs) and OAHFAs; sphingolipids (sphingomyelin [SM] and ceramide [Cer]); glycerophospholipids (lyso-phosphatidylcoline [LPC], lysophosphatidylethanolamine [LPE], and phosphatidylcoline [PC]); glycerolipids (triglyceride [TG] and diglyceride [DG]); sterol lipids (cholesteryl ester [CE]); and WEs.

The most intense ion for PC, LPC, SM, and WEs was $[M+H]^+$, whereas it was $[M-H]^-$ for the FAs and OAHFAs (Table A2). As expected, the addition of ammonium formate to the positive ion mode mobile phases enhanced the signal of TG, which was detected as $[M+NH_4]^+$. Despite the use of ammonium formate, some sodium adducts [M+Na]+ were also detected. In the case of Cer, DG, and CE, this sodium adduct was the most intense detected ion, whereas for PC, LPC, and SM, it was the second most intense ion after the protonated molecular ion, and for TG, the second most intense ion after $[M+NH_4]^+$. In the case of Cer, DG, and CE, a sodium adduct was the most intensely detected ion, whereas for PC, LPC, and SM, it was the second most intensely ion detected after the protonated molecular ion.

To evaluate the coverage of the two methods in the ESI+ and ESI– modes, a PCA was performed on the data set of the 69 lipids and 25 samples (ten replicates for each sample preparation method and five QC samples). The plots showed a separation between extraction and precipitation (Figure 3A,B), and we investigated the main sources of the variation in the PCA by inspecting the loading plots (Figure 3C,D). The loading plots showed that the main difference between the methods was due to the specific interaction of the different lipid classes with the extraction solvent based on their solubility. Thus, BD extraction resulted in an increase in TG, DG, WE, CE, and FA, whereas IPA precipitation considerably enhanced the ability to study LPC, LPE, PC, and SM.

*Assessment of recovery:* The efficiency of the BD and IPA protocols was tested in terms of the capacity to quantitatively recover 20 non-endogenous ISs spiked into the samples before and after recovery (Figure 4). The lipid standards selected for this purpose represent the major lipid families present in the tear samples (glycerophospholipids, glycerolipids, sphingolipids, FAs, OAHFAs, WEs, and sterol lipids), and their recovery was calculated as the ratio of the peak areas of the ISs in the samples spiked before extraction relative to those added after sample preparation.

The main differences between the two methods analyzed (BD and IPA) appeared to be predominantly due to the intrinsic differences in the lipid selectivity of the solvents used. Thus, polar lipids (PC, LPC, LPE, PE, SM, and Cer), except OAHFAs, were better recovered with the IPA method than with the BD extraction method (Figure 4). Thus, the BD method produced a loss of lysophospholipids, phosphatidylglycerol (PG), and phosphatidylinositol (PI), as demonstrated by their poor recovery (<45%). In contrast, although the recovery of non-polar lipids (TG, DG, WE, and CE) and the polar OAHFA was a little better using the BD approach, the difference between the two methods for these classes of lipids was not statistically significant. Therefore, IPA precipitation produced highly consistent and reliable recovery (>62–100%) of the 20 lipid standards tested, indicating that this approach produced better recovery and coverage than the traditional BD method for tear samples.

*Reproducibility:* Untargeted lipid profiling is based on the relative comparison of spectral profiles, and therefore, it is necessary to employ a systematic QC strategy to assess the reproducibility of the analytical workflows. The IPA and BD protocols were tested for their ability to produce similar profiles from replicates analyzed under the same conditions. Thus, a pooled tear sample was split into 20 aliquots. Ten of which were extracted independently with the BD method, and the other ten were precipitated using the IPA method. Stable retention times were obtained after the column was conditioned with multiple (n = 15) injections of QC samples. The typical variation in the retention times under the lipid profiling conditions did not exceed 2.4 s, and it did not exceed 1.2 s in the case of the variation in mass, which was critical for correct data preprocessing and feature identification. Histograms of the peak intensity CVs were used to visualize the distribution of features (4,232 in ESI+ and 382 in ESI–) in the QC replicates (RT and *m/z*) and those of the samples from the two extraction protocols. Each histogram represented the dispersion of the CV values for the features from one condition (Figure 5). We found that 99.5% of the features from the pooled QC had a CV below 20%, which indicates significant consistency of the instrument and samples over time, and it represents an acceptable value in biomarker analysis. The CVs obtained with IPA precipitation showed this to be a

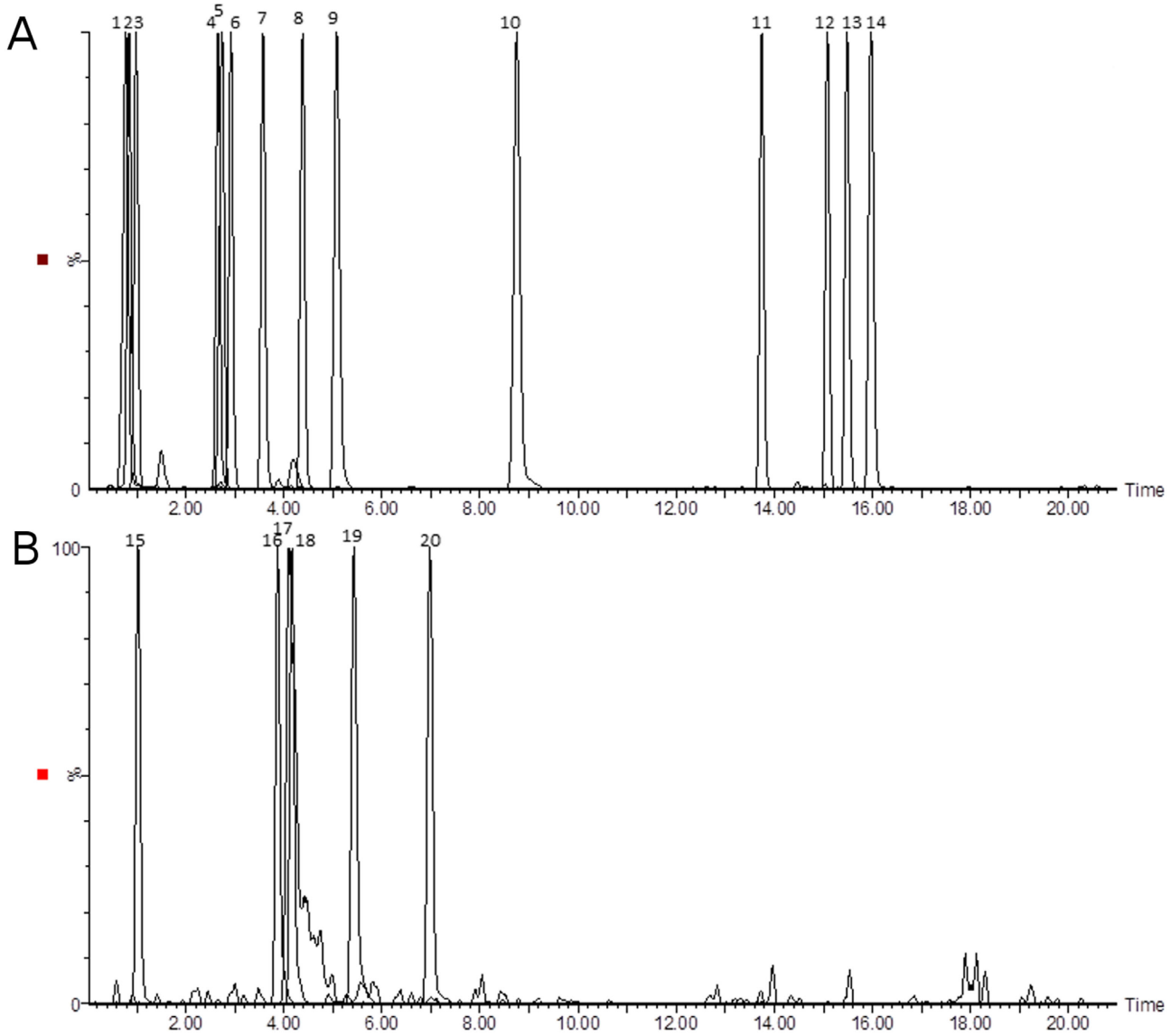


Figure 2. Ion Chromatograms of the internal standards. Extracted ion chromatograms of the internal standards detected either in the positive (A) or in the negative (B) ion mode. 1: Sphingosine (C17); 2: Sphinganine (C17); 3: 18:1(d7) Lysophosphatidylcholine (Lyso PC); 4: Sphingomyelin (C12); 5: Lactosyl(β) C12 Ceramide; 6: Glucosyl(β) C12 Ceramide; 7: Ceramide (C12); 8: d18:1–18:1(d9) SM; 9: 15:0–18:1(d7) PC; 10: 15:0–18:1(d7) DG; 11: Ceramide (C25); 12: 15:0–18:1(d7) −15:0 TG; 13: 18:1(d7) Chol Ester; 14: 18:1(d9)-26:0 WE; 15: 18:1(d7) Lyso PE; 16: 15:0–18:1(d7) PI (NH4 Salt); 17: 15:0–18:1(d7) PS (Na Salt); 18: 15:0–18:1(d7) PG (Na Salt); 19: 15:0–18:1(d7) PE; and 20: 18:1–16:0 OAHFAs.

robust lipid extraction method, because 73.5% of the features in ESI+ and 68.8% in ESI– had a CV below 20%, whereas with the BD protocol only 26.8% of the features in ESI+ and 35.6% in ESI– had a CV lower than 20%. Thus, the BD method appeared to be less repeatable. When we compared the %CV distributions between the three groups (QC, BD, and IPA) with the Kruskal–Wallis test, the two methods for sample preparation differed statistically significantly and from the QC ($p<0.01$).

*Linearity and sensitivity:* Due to the different concentration ranges of the analytes in the lipid samples, it is essential that the analytical methods cover a large dynamic range, and they provide sufficient sensitivity to permit the quantification of molecules at low and high concentrations. To assess these parameters, increasing amounts of lipid ISs were added before sample preparation, and highly linear calibration curves were obtained ($R^2$=0.97–0.99), enabling the correlation between the acquired signal and the amounts to be calculated (Table

1). Sensitivity was given by the calibration curve slope, and although the steepest slope was obtained for ceramide C12, 18:1(d7) CE had the lowest sensitivity. The LOD and LOQ values were calculated as those corresponding to the signal-to-noise ratios of 3:1 and 10:1, respectively. The lowest LOD and LOQ values were obtained for Cer (C12: LOD 0.02 µg/ml, LOQ 0.03 µg/ml), and the highest values corresponded to 18:1 (d7) CE (LOD 0.47 µg/ml, LOQ 2.82 µg/ml; Table 1). The quantification of these limits showed that the IPA precipitation method was sufficiently sensitive to determine the lipid compounds in tear samples (Figure 6).

## DISCUSSION

Different solvent extraction systems have been proposed for lipidomic studies; specifically, Folch [20] and BD [21] are widely used. The Folch extraction protocol was originally established to isolate lipids from brain tissue using chloroform:methanol:water, while the BD extraction method involves a reduction in the amount of chloroform used in Folch extractions. These two methods are popular because the combination of methanol and chloroform non-selectively and reproducibly extracts a broad range of lipid classes from a wide variety of matrices. More recently, methyl tert-butyl ether (MTBE) extraction was proposed as an alternative solvent extraction protocol for lipidomics, providing comparable results to the Folch and BD methods for plasma and *Escherichia coli* but without involving the use of chloroform [27]. These three liquid-liquid methods are based on the extraction of diverse lipid classes using a binary mixture of methanol with a non-polar organic solvent (chloroform or MTBE). With the range of solvent extraction systems available in the literature, and given that each method can be modified by the use of buffers, salts, or antioxidants, matrix-dependent optimization is necessary to ensure the optimal extraction of lipids in a reproducible manner. For this reason, two lipid extraction methods were evaluated in the present study for untargeted lipid profiling with UHPLC-QTOF of a limiting volume of tear fluid. The methods analyzed were a traditional BD chloroform-based lipid extraction procedure and an IPA protein precipitation method, comparing a set of quantitative criteria that included lipid coverage, repeatability, and efficiency of recovery, as well as qualitative criteria such as simplicity and adequacy for large-scale analysis. In addition, these methods were used to identify and quantify lipids in tear samples collected from healthy subjects. In particular, we focused on the simplest and most reproducible sample preparation method and with the most affinity for the polar lipids for tear lipidomic analysis, because our future research will be the focus of the analysis of this lipid class.

Taking into account that the BD method uses a chloroform:methanol mix, the collection of the lipid fraction requires a careful pipetting of the dense, lower chloroformic phase, avoiding contamination with proteins at the interface, which could diminish performance. However, protein precipitation protocols involve monophasic solvent mixtures, producing a protein pellet at the bottom of the tube, and thus, avoiding any problems associated with the presence of an interface, decreasing the risk of proteins contaminating the lipid extract. Manual pipetting was used in the present study, steps that will be critical when automated preparation of samples is considered. The resuspension of the dried extracts is another critical step, as the BD extraction protocol analyzed implies reconstitution of the dried material with a solvent mixture close to the initial mobile phase, which is not necessary in the IPA protocol. This avoids the poor chromatographic separation of the lipid classes associated with the direct injection of chlorinated solvents. Such solvent exchange prolongs the sample preparation time and increases the risk of introducing procedural errors.

The recovery of lipids from the tear samples was similar with the two methods analyzed, with the differences observed mainly associated with the intrinsic lipid selectivity of each solvent. Polar lipids, except OAHFAs, were better extracted with the IPA method than in the BD method, while the BD protocol also provoked some lysophospholipid loss. Recovery of the non-polar lipids (TG, DG, WE, and CE) and polar OAHFAs was similar with both methods, with a slight improvement when the BD protocol was used. The improved recovery observed with IPA precipitation was consistent with a previous study on plasma samples, in which this protocol produced highly consistent and reliable recovery (>60−80%) of the lipid standards tested [22]. In terms of reproducibility, the IPA protocol also appeared to be more robust than the BD protocol, although it differed statistically significantly from that of the QC. This was not in accordance with the data from the analysis of plasma samples where no differences were evident between IPA and the intrinsic instrumentation reproducibility, possibly reflecting the lower intrinsic reproducibility seen in this previous study compared to that seen in the present study [22].

Likewise, the unsupervised PCA clearly indicated that the two methods for isolating lipids from tear samples generated different LC-MS profiles. The data were much better grouped when lipids were extracted using the IPA protocol; the BD protocol produced greater dispersion and variability of the data. This variability is likely due to the higher number of steps in the BD extraction protocol. Moreover, the QC

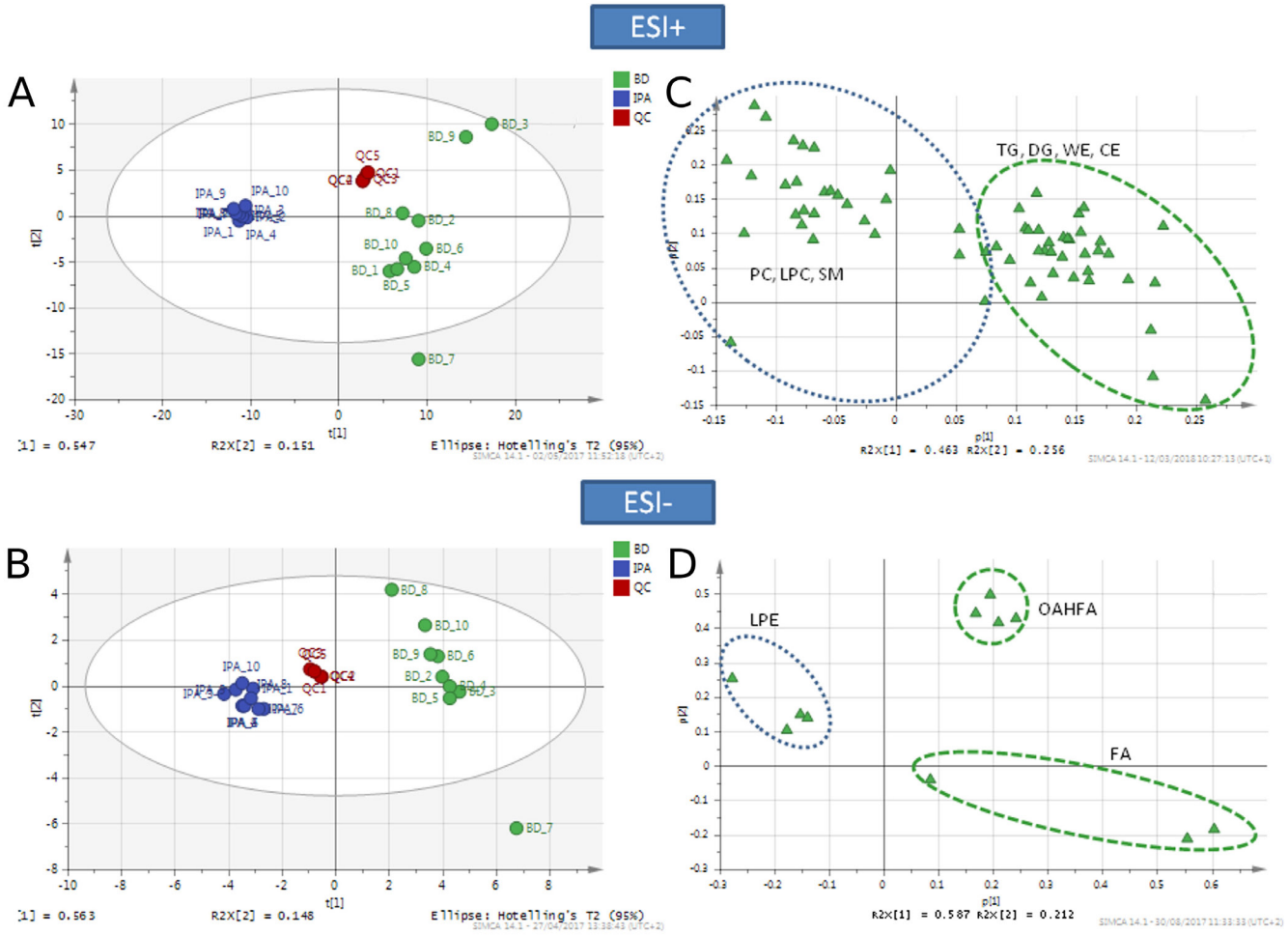


Figure 3. The PCA analysis indicates intrinsic differences between the groups. Score plots (A and B) and loading plots (C and D) obtained from BD extraction and IPA precipitation samples analyzed with lipid profiling in the positive and negative ion modes. The isopropanol precipitation (IPA) method leads to tighter clustering of the samples while the Bligh and Dyer (BD) extraction protocol exhibits larger variability.

profiles were clustered tightly in the PCA plots, demonstrating the reliability of the UHPLC-QTOF-MS platform.

We applied what are sensitive quantitative methods to analyze small tear volumes, enabling the lipid profile of the tear in healthy individuals to be established. The major lipids identified in the tears were CE 24:0, WE 18:1/26:0, TG 49:2, SM 18:1/16:0, OAHFA 18:1/32:1, and LPC 16:0. These results are consistent with previous data, in which CE represented 39–45% of the lipids in tear fluid and 44% in the meibum [6,12]. Recently, human meibum and aqueous tears were seen to differ in the molar ratio of lower molecular weight WE-like compounds [28]. High performance liquid chromatography-multiple reaction monitoring (HPLC-MRM) approaches measured the WE molecular species in human tears, establishing a total of 141 WE molecular species in the tear WE profiles [23]. Quantitative analysis indicated that oleic acid (18:1) containing species are the most abundant WE present in tears. The WE composition of this fluid is important, as it is a film that avoids and prevents evaporation [29,30]. In contrast, amphiphilic lipids (such as OAHFAs, some sphingolipids, and phospholipids) interact with protein components of the tear playing a key role in maintaining the tear surface tension. These components are important for the physiologic hydration of the ocular surface and ocular homeostasis [31]. This amphiphilic layer was seen to be notably enriched in phospholipids and sphingolipids relative to the meibum, and there is a surplus of phospholipids, lisophospholipids, and sphingolipids in tears to constitute the amphiphilic lipid sublayer. Due to the affinity of isopropanol for amphiphilic lipids, we found a great abundance in LPE, LPC, PE, PG, PI, and PS; all of these notable lipids should be further evaluated in pathologies of the ocular surface as the DES in which the

lipid composition of the tear is altered. We detected the same lipid classes with the two methods, but the difference in these lipid amounts is important to emphasize.

A detailed evaluation of different sampling techniques could help standardize analytical protocols, thus facilitating future biomarker studies on tears. We defined a simple, efficient, and reproducible method for isolating lipids from small tear samples, opening up possibilities for semiautomatic analysis of large numbers of specimens. Additionally, the use of this procedure may help identify lipid biomarkers that could better characterize defined eye diseases and hopefully, promote the design of artificial tears adequate for each pathology.

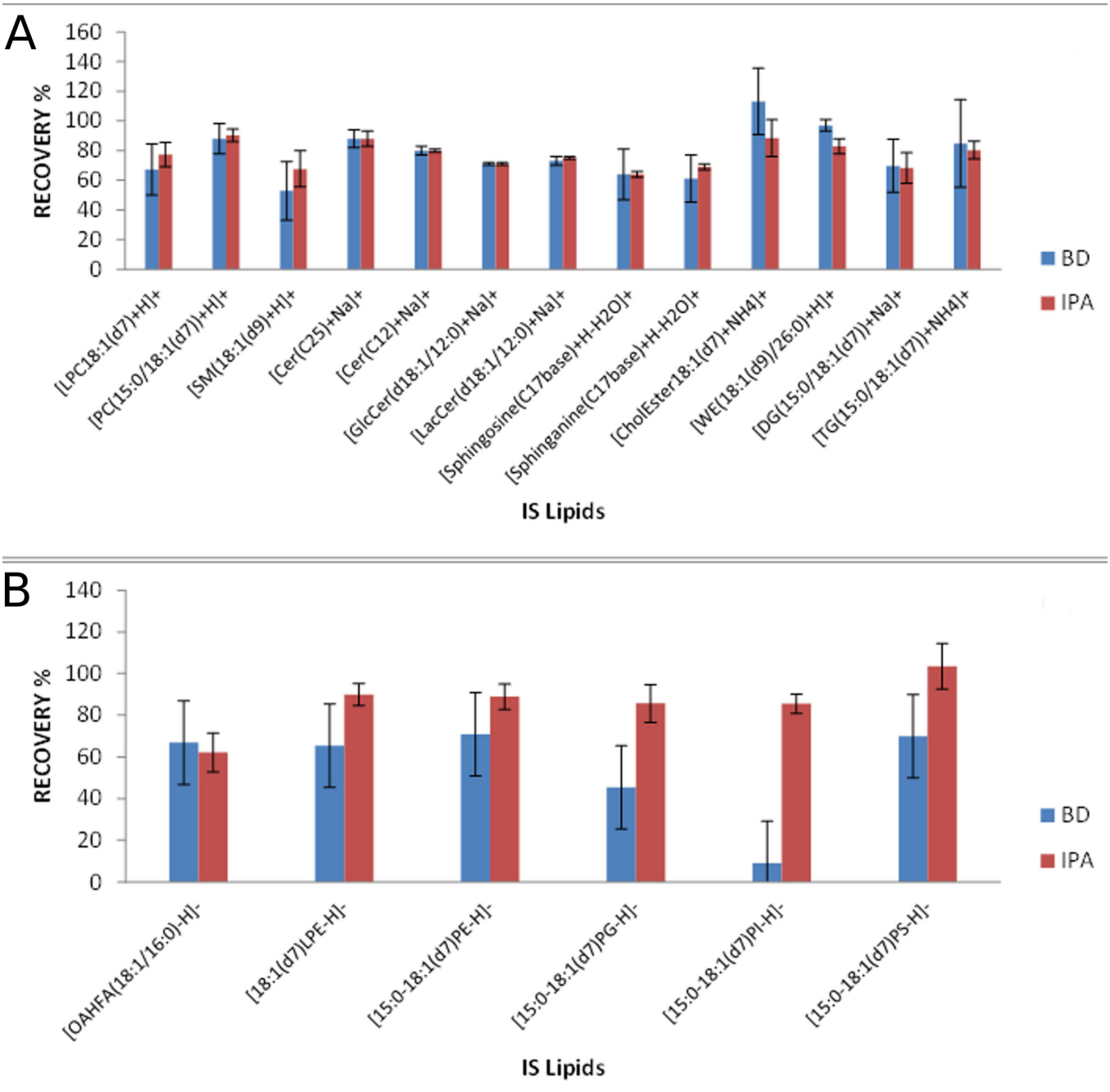


Figure 4. Lipid recovery of sample preparation methods. The recovery percentage of BD and IPA extraction methods for each lipid class according to the ion mode detected: ESI+ (**A**) and ESI– (**B**). Mean ± standard deviation (SD).

*CONCLUSIONS:* We evaluated and compared two methods to prepare samples for tear untargeted lipid profiling with UHPLC-MS, assessing their lipid coverage, repeatability, and recovery efficiency, as well as their simplicity of execution. The BD method requires technical skills and involves twice as many steps (four) as the IPA method (two). Repeatability is one of the most important criteria to improve the reliability of an analytical assay, aiding the identification and use of potential biomarkers, and IPA outweighs BD. We conclude that protein precipitation with IPA is an easy, efficient, and reproducible method for extracting lipids from minimum tear volumes, which will enable the quantification of lipids in the tears of patients with ocular surface pathologies.

## APPENDIX 1: LIST OF LIPID STANDARDS USED IN THE CURRENT STUDY.

To access the data, click or select the words "Appendix 1."

## APPENDIX 2: OAHFA STANDARD (A,C) AND WE STANDARD (B,D) WERE CHARACTERIZED BY $^{1}H$ AND $^{13}C$ NMR SPECTROSCOPY AND BY HRMS. THEIR PURITY WAS ESTABLISHED AS >95%.

To access the data, click or select the words "Appendix 2."

## APPENDIX 3. CHROMATOGRAPHIC GRADIENT USED FOR LIPID PROFILING.

To access the data, click or select the words "Appendix 3."

## APPENDIX 4. LIST OF LIPID STANDARDS USED FOR THE RECOVERY STUDY AND THE MOST INTENSE ION PEAK FOR EACH INTERNAL STANDARD.

To access the data, click or select the words "Appendix 4."

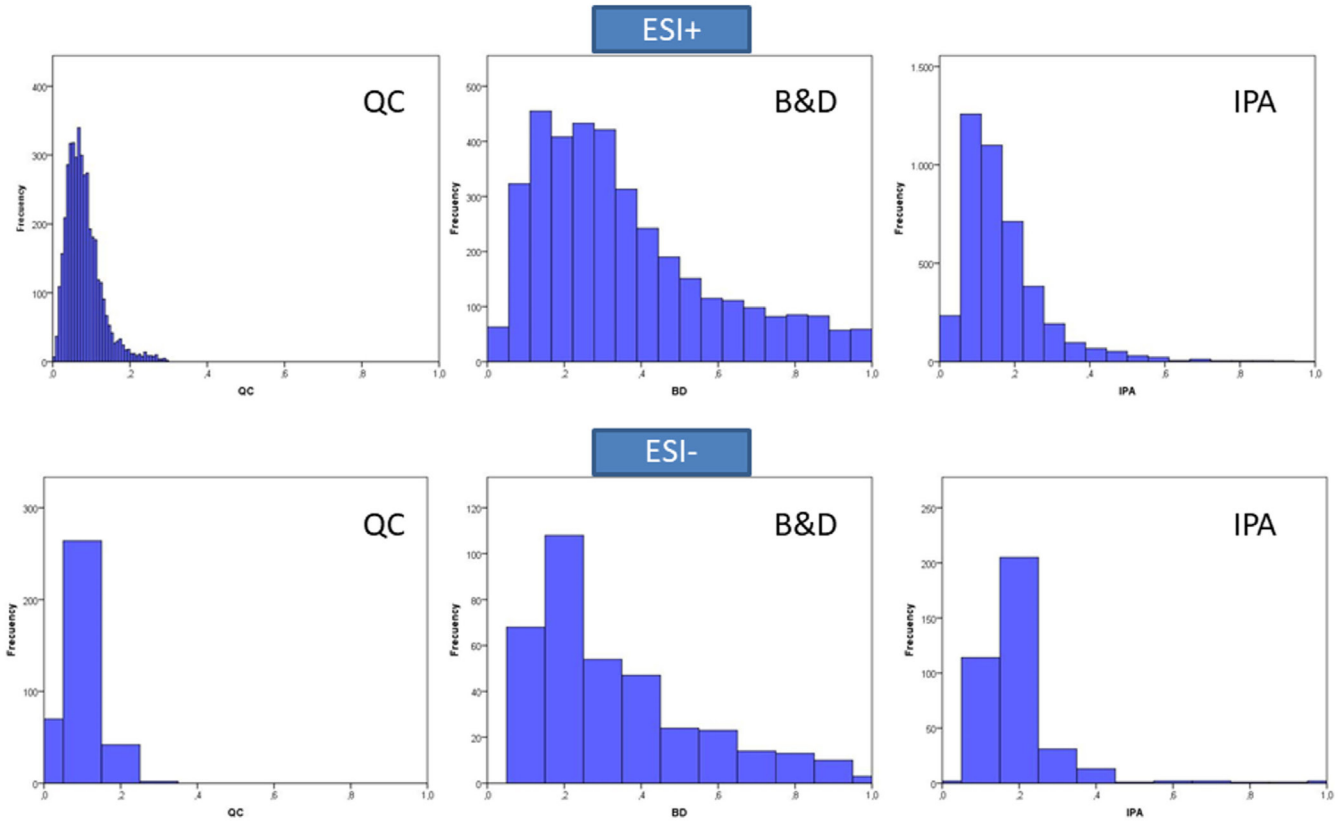


Figure 5. Histograms of the CVs to assess the reproducibility of the two sample preparation methods. Histograms of BD and IPA methods. The histograms of the CVs were used to assess the reproducibility of the two lipids extraction methods comparing with a quality control (QC) in ESI+ and ESI-.

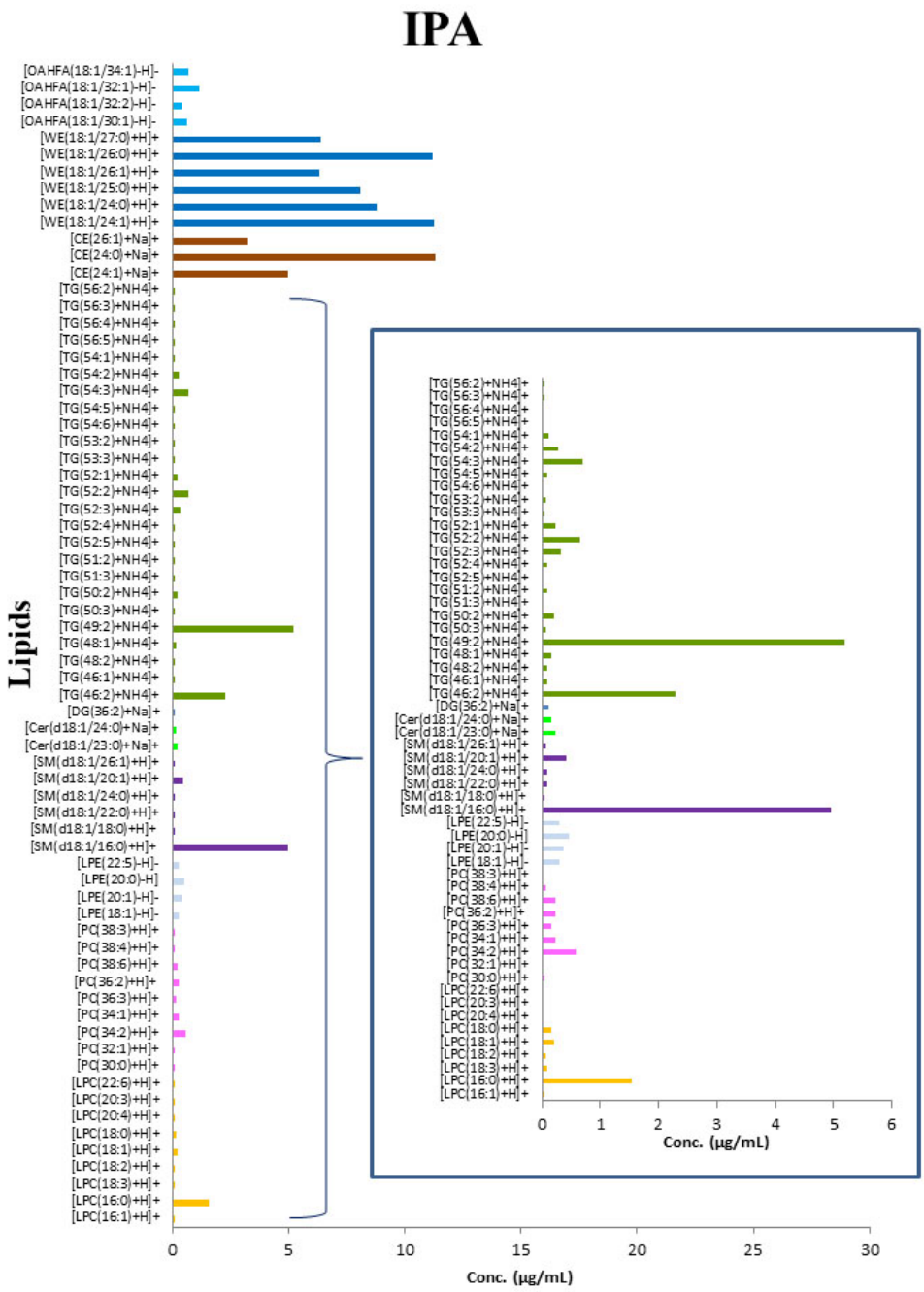


Figure 6. Schematic summary of the tear lipid composition quantified with UHPLC-MS on samples prepared with the IPA protein precipitation method.

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