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# Effective methods for the investigation of human tear film proteins and lipids

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**Abstract.** The investigation of human tear film proteins and lipids is of value for the elucidation of contact lens incompatibilities, tear film instabilities, dry eye syndrome and various other eye diseases. Improved efficient methods for the investigation of human tear film proteins and lipids are presented in this paper. Tear proteins were examined by ultrathin sodium dodecyl sulfate-polyacrylamide gel electrophoresis, celluloacetate gel, isoelectric focusing, and high-performance liquid chromatography. The methods differ in sensitivity, resolution, convenience and reproducibility. Tear lipids were examined by high-performance thin-layer chromatography, and good separation into the major lipid classes was achieved. With this method it is possible to examine the lipids present in tears of an individual subject and not just in pools made up from the tears of several persons.

## Introduction

Proper function of the tear film is essential for the eye. The tear film humidifies and nourishes the cornea, protects the eye against external infection and contributes to the refraction of the light path. The tear film is composed of three layers. The superficial oily layer (0.1–0.2 μm thin), which prevents evaporation, the middle aqueous layer (5–7 μm thin), which contains enzymes, minerals and proteins for the nutrition of the cornea and the antibacterial defense, and the deep mucous layer (0.02–0.04 μm thin) in which mucins are located, which prevent the break-up of the tear film [16, 36, 37].

We are especially interested in the investigation of lipids and proteins and have therefore developed the methods described below.

### *Proteins*

The tear film proteins are produced by the lacrimal gland and the accessory lacrimal glands of Krause and Wolfring. Tear proteins have already been investigated by various electrophoretic methods, by isoelectric focusing (IEF), by high-performance liquid chromatography (HPLC) [5] and by two-dimensional gel electrophoresis [12]. Single tear proteins have been quantified by enzyme-linked immunoadsorbent assay (ELISA), radial immunodiffusion and electroimmunodiffusion [25, 31]. Up to 60 protein components have been found by two-dimensional electrophoresis [12], four of which are found in higher concentrations, namely lactoferrin (an iron-binding protein with bacteriostatic effect), lysozyme (a bacteriolytic protein with a concentration in tears that is higher than in any other body fluid), "proteins migrating faster than albumin" (PMFA), a tear-specific prealbumin that has been found only in tears [4], and albumin (identical to serum albumin) [36]. Investigations of the tear protein pattern would be of value for study of the blood-tear barrier [24], in keratopathy, conjunctivitis, glaucoma, inborn metabolic diseases, contact lens incompatibilities and after drug administration [23].

### *Lipids*

The tear film lipids are produced by the meibomian glands and the glands of Zeis and Moll. Mostly, the lipids of the meibomian glands have been examined so far, because they are available in greater quantities than tear film lipids. Tear film lipids have been preferably examined in pooled samples because of their small quantity. The methods used have been thin-layer chromatography (TLC) and gas chromatography [34]. In pooled samples of meibomian secretion and tears, the main lipid classes have been found to be wax and cholesterolesters and cholesterol. Triglycerides, free fatty acids and phospholipids have been found in smaller quantities and not in all patients [1, 11, 34]. In meibomian lipids, significant differences in the relative amounts of the lipids have been found in samples of different patients [34]. Screening of tear film lipids would be interesting not only in the investigation of normal persons to establish normal tear lipid patterns, but also in blepharitis, keratopathy, dry eye syndrome [19] and contact lens incompatibility.

## Materials and methods

### *Tear collection*

Tears were collected by a microcapillary (with some gentle mechanical stimulation) from the inferior fornix of the conjunctiva from healthy persons and a few cases with conjunctivitis.

### *Total protein*

The total protein content was determined according to Bradford [7] using a mixed standard (70% globulin, 30% albumin); 1 μl of tear fluid was used per analysis.

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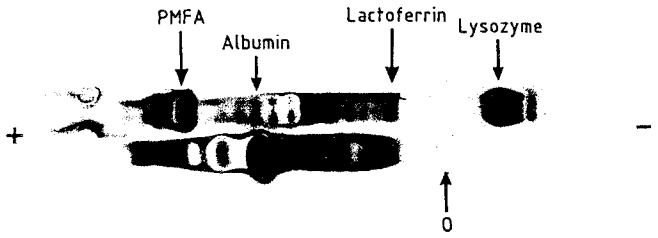


**Fig. 1.** Electrophoresis on celluloacetate gel, using *denaturing* buffer conditions. Gold stained. Samples: *above* 0.25 µl tear fluid; *below* 0.25 µl serum. *O*, Origin

*Celluloseacetate gel electrophoresis (CAG)*

CAG was performed in a Beckman microzone chamber; 1–2 µl of tear fluid was necessary. Under *denaturing* conditions, the reduced proteins were separated using the following buffer: 12.11 g TRIS (hydroxymethyl)aminomethane, 16.32 g bicine, 3.5 ml mercaptoethanol, 480.48 g urea, pH 8.6, and distilled water to 1000 ml [28, 33, 39]. The running conditions were: 350 V, 4 mA, for 70 min; *nondenaturing* separation conditions were used according to Serva [32]. The buffer composition was: 8.24 g diethylbarbituric acid sodium-salt, distilled water to 1000 ml, pH set at 9.7 by HCl. The running conditions were: 210 V, 4 mA, for 40 min. After electrophoresis, the CAG strips were fixed for 30 min in 12.5% trichloroacetic acid (TCA), stained for 1 h in Coomassie blue (staining solution: 70 ml acetic acid, 10 g Coomassie blue R 250, distilled water to 1000 ml) and made transparent according to Chemetron [18]. After Coomassie blue staining micrograms of protein can be detected, quantification is possible by scanning at 570 nm. For identification of the major protein bands, the CAG strip was equilibrated after electrophoresis in 20 ml buffer [60.6 g/l TRIS (hydroxymethyl)aminomethane buffer, 1 g/l sodium dodecyl sulfate], polymerized in a stacking gel and run in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as a second dimension [2]. Identification, especially of the minor proteins, can also be done by immune CAG [26]. In addition, gold staining was performed according to Chemetron [9] to detect minute traces (nanograms) of protein (Fig. 1).

*SDS-PAGE in 0.75-mm thin gels*

Polyacrylamide gels 0.75 mm thin (gradient 5%–25%) were molded on Gel Bond PAG (LKB, Bromma, Sweden) with a 5% stacking gel on top. The gels were run horizontally on a LKB-Multiphor [15, 17]. The running buffer was: 3 g/l TRIS (hydroxymethyl)aminomethane, 14.5 g/l glycine, 1 g/l SDS. The running conditions were: 600 V, 40 mA, 20 W, for 2 h. In each lane, 0.5 µl tear fluid (=4 µg protein) was applied. Up to 24 samples were run on one gel simultaneously. After electrophoresis, gels were fixed in 20% TCA for 15 min and stained for 1 h in a 0.2% Coomassie blue solution containing 2 g Coomassie blue R-250, 75 ml acetic acid, 500 ml methanol, and distilled water to 1000 ml. Destaining was done overnight in a solution containing 7.5% acetic acid and 50% methanol. The gel was scanned at 570 nm. For detection of nanograms of protein the gels were silver stained according to Merril et al. [27]. Identification can be made by coelectrophoresis of purified proteins or by Western blotting according to Towbin et al. [35], using antisera against the single proteins [26]. Since the major tear proteins in polyacrylamide gels have already been identified by other authors, we followed their identifications [12, 26].

*Isoelectric focusing*

IEF was performed in 0.7-mm thin slab gels. The gel composition was: 5% polyacrylamide, 20% glycerol, and 10% ampholytes (from SERVA, pH range 2–11). Gels were cast on Gel Bond films (from LKB) between silanized glass plates using 0.7-mm spacers. IEF was carried out in a LKB Multiphor apparatus at 4° C. One-centimeter electrode strips, which were soaked in 1 M NaOH at the cathode and in 1 M $H_3PO_4$ at the anode, were placed at the ends of the gel. Samples were applied by means of a silicone rubber strip 3 cm from the cathode; 2–3 µl tear fluid was applied per lane. The running conditions for prefocusing were: 590 V, 33 mA, 19 W for 45 min (0.64 kVh); for focusing: 1200 V, 4 mA, 7 W for 2–3 h (2.84 kVh). Afterwards, IEF gels were fixed in 12.5% TCA and then stained for 2–3 min in a solution containing 2 g/l Coomassie blue R-250, 10% acetic acid, 45% ethanol. Destaining was done in a solution containing 20% ethanol, 8% acetic acid. The gel was scanned at 570 nm. Identification was done by coelectrophoresis of the main proteins, which had been purified before in a non-denaturing polyacrylamide gel without SDS.

*High performance liquid chromatography*

Tear proteins were eluted isocratically on a HPLC column (TSK 3000 SW, 600 × 7.5 mm, LKB) with 0.1 M ammonium acetate buffer, pH 4.1, as eluent buffer and a flow rate of 1 ml/min; 1 µl of tears was necessary. The detector was set at 230 nm, 0.01 absorbance. One sample could be examined in 20–30 min. Identification was done by running the eluted fractions on SDS-PAGE. For further details see [3].

*Total lipids*

Total lipids were determined according to Zöllner and Kirsch by the sulphophosphovanilline reaction [38]; 1 µl of sample was used per analysis.

*High-performance thin-layer chromatography (HPTLC)*

HPTLC was performed in a modification of Kupke's and Zeugner's method for serum lipids [20]; 15–20 µl tear fluid was necessary per sample. We used HPTLC silica gel plates with a concentrating zone from Merck (Darmstadt, FRG) and a size of 20 × 10 cm or 10 × 10 cm. The solvents were HPLC grade from Merck. The HPTLC plates were cleaned overnight in solvent solution I (see below). Afterwards, the solvent was evaporated and the plates activated for 1 h at 110° C. For lipid extraction, the tear samples were mixed with half their volume of methanol and centrifuged in polystyrene tubes before application on the plates. The samples were applied on a spot as small as possible under a continuous cold air stream. Up to 7 samples were applied on the 10 × 10 cm plate and up to 14 on the 20 × 10 cm plate.

After drying, the HPTLC plates were placed in a chamber with a saturated atmosphere of the solvents and developed as follows: two times up to exactly 4 cm from the lower edge of the plate by solvent solution I: chloroform, methanol, distilled water, 65:30:5. This procedure

took about 2 × 5 min. Afterwards, two times up to exactly 0.5 cm from the upper edge of the plate by solvent solution II: *n*-hexane, diethyl ether, acetic acid, 80:20:1.5. This procedure took about 2 × 13 min. Afterwards, the plates were sprayed with a 25% sulfuric acid solution for the detection of the lipids. Then the plates were immediately placed into an oven (110° C) for at least 1 h. Afterwards the lipids were visible. Identification was done by comparing the tear lipids with the known separation pattern for serum lipids [20]. Quantification can be done visually by comparing the size and the intensity of the tear lipid spots with the known lipid concentration of a lipid standard mixture.

## Results

*Normal value for total tear protein*

The mean value for 20 normal persons was 8 ± 2.25 g/l, which is in good accordance with values in the literature [14].

*Cellulose acetate gel electrophoresis*

Under *denaturing* conditions, tear proteins were separated from cathode to anode into the four main fractions, lysozyme, lactoferrin, albumin and PMFA. Above the albumin, two additional faint bands could also often be found by Coomassie blue staining. Gold staining revealed 16–18 bands (Fig. 1). Under *nondenaturing* conditions tear proteins were separated into three bands, from anode to cathode lysozyme, lactoferrin, albumin and PMFA in one band [21]: results not shown.

*Ultrathin SDS-PAGE*

Tears were separated from cathode to anode into immunoglobulins (IgG), lactoferrin, albumin, protein G (13, 14), PMFA and lysozyme (Fig. 2). In healthy persons, immunoglobulins could not always be detected in contrast to cases with conjunctivitis. Silver staining revealed further bands, especially in pathological tears.

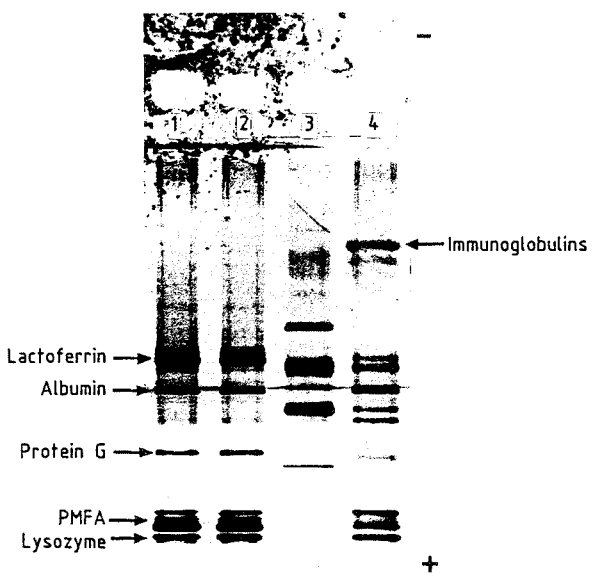


**Fig. 2.** Ultrathin sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5%–25%). Silver stained. *Samples*: *Lanes 1 and 2*: 2 µl tear fluid: *lane 3*: 2 µl molecular weight standards (from Serva/95, 68, 45, 29 kDa); *lane 4*: 2 µl pathological tears. Immunoglobulins (IgG) were usually found mostly in pathological tears

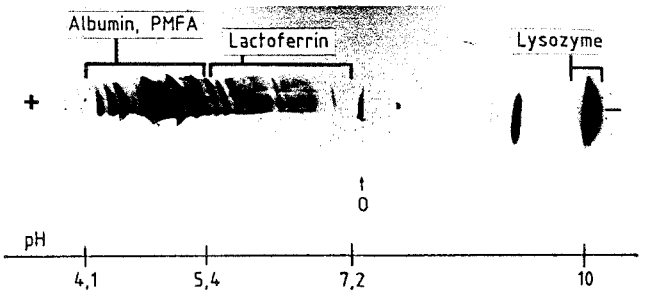


**Fig. 3.** Isoelectric focusing (pH range 2–11) of tear proteins. *Samples*: 3 µl human tear fluid. Stained by Coomassie blue R-250. *Below*: pH scale. *O*, Origin

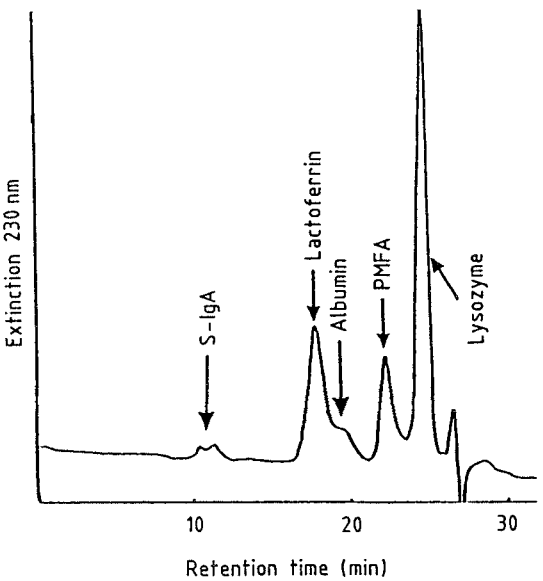


**Fig. 4.** Elution profile of tear proteins on high-performance liquid chromatography column. *Sample*: 1 µl human tears. *Column*: TSK 3000 SW. Solvent: ammonium acetate buffer (pH 4.1)

*Isoelectric focusing*

Tear proteins were separated into 21 bands. From anode to cathode: albumin, PMFA, lactoferrin and lysozyme (Fig. 3). Most proteins indicated several bands due to their microheterogeneity, which could also overlap with the bands of other proteins and were very close so that the identification given here is possibly not absolutely exact.

*High performance liquid chromatography*

Tears were separated into immunoglobulins (S-IgA), lactoferrin and albumin, PMFA and lysozyme (Fig. 4). Albumin was not completely separated from lactoferrin (Fig. 4) [3].

*Normal value for total tear lipid*

The mean value for 20 normal persons was 1.8 g ± 0.8 g/l.

*High-performance thin-layer chromatography*

A good separation of tear lipids into at least three phospholipids, cholesterol, fatty acids, triglycerides and cholesterol/

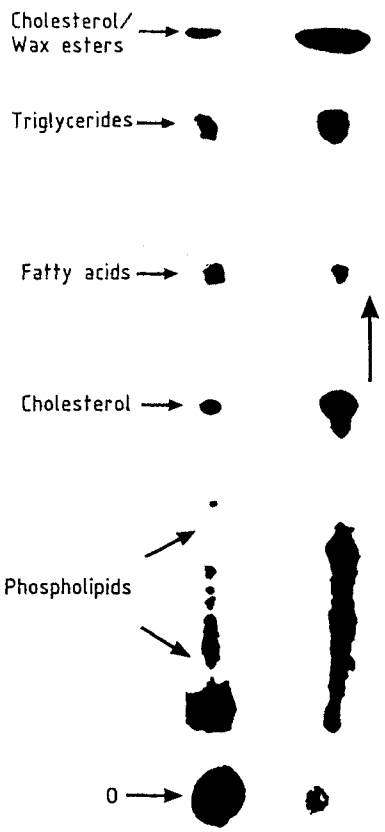


**Fig. 5.** Silica gel high-performance thin-layer chromatography plate developed by the consecutive use of two different solvent solutions. *Samples*: *left* 15 µl human tears; *right* 10 µl human serum. *O*, Origin with the non-lipid components (proteins, mucins). *Arrow* shows direction of solvent flow

wax esters, was achieved (Fig. 5). Cholesterol esters and wax esters could not be separated. Phospholipids (15%), cholesterol (15%) and cholesterol/wax esters (45%) showed higher concentrations than the other lipids. However, there were individual differences and phospholipids, for example, were not always detectable.

## Discussion

*Denaturing CAG electrophoresis* showed a better resolution than the commonly used nondenaturing buffer systems, because albumin and PMFA were clearly separated. Therefore, we recommend this denaturing buffer. CAG is a relatively cheap, convenient and reproducible screening method for the quantitative determination of the four main proteins, but less suitable for the other proteins in minor concentrations because these can only be detected by gold staining, which cannot be quantified [9].

*Ulrathin SDS-electrophoresis* (SDS-PAGE) yielded a good resolution of most tear proteins, including the so-called minor proteins, as already shown by Mur et al. [28]. Silver staining proved especially suitable for the detection of minor proteins, but is only semiquantitative in contrast to Coomassie blue staining. Therefore, we recommend conducting first Coomassie blue staining for quantitative analysis and then silver staining to detect additional bands that may have special diagnostic importance. SDS-PAGE is a sensitive and convenient method for the separation of the tear proteins and is especially suited for screening because up to 24 samples can be run simultaneously on one gel.

By means of *IEF* the tear proteins were separated into 21 bands. However, due to microheterogeneity, one protein can show several bands that may even overlap with bands from other proteins, and the bands are so close to one another that densitometric evaluation is difficult.

*HPLC* showed good resolution of the major tear proteins except for albumin and lactoferrin. HPLC can be completely automated and quantified by using an autosampler, a detector and an integrator and is therefore especially suited for routine application.

*HPTLC* showed a good resolution of the tear lipids. In contrast to normal TLC, relatively small amounts of tears are necessary, so that screening of tear film lipids should become possible. By UV scanning it should be possible to reduce the amount of tears required even more and to quantify exactly the components of the tear film lipids [30].

In conclusion, the described methods should be suitable for large-scale screening of tear film proteins and lipids. Our future interest is first to establish the typical tear protein and lipid patterns for various ocular and systemic diseases that may be helpful for the diagnosis, treatment and elucidation of pathogenetic mechanisms of these diseases. Secondly, we are trying to find a correlation between certain protein [22, 24] and lipid variations and the development of specific contact lens deposits, which can lead to contact lens incompatibilities [6, 10, 18, 29]. In this way, it should be possible to recommend a suitable type of contact lens to patients by analyzing their tear fluid before adaptation, thereby avoiding unnecessary loss of time, money and even damage to the eye.

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