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Analysis of Human Tear Fluid by Fourier Transform Infrared Spectroscopy

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Abstract: *The purpose of this research is to find some useful spectroscopic factors in human tear fluid contents to monitor diurnal changes of the physicochemical ocular conditions noninvasively. All tear fluid samples were collected with glass microcapillary tubes from both eyes of three donors and analyzed by Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR–ATR). We measured the peak intensities at 2852, 1735, 1546, and 1242 cm^{-1}, and the peak intensity ratios among those peaks in the second derivative spectra. We found significant diurnal and individual variations in those peak intensities for tear fluid obtained from right and left eyes. Among these variations, we observed significant changes in tear samples between right and left eyes. In this case the peak intensity ratio between 1242 (phosphate ester) and 2852 cm^{-1} (fatty acid methylene) of right eye tear fluid was increased in the afternoon (1600 to 1900 h), while that of left eye tear fluid did not change significantly. In the ratio between 1242 (phosphate ester) and 1546 cm^{-1} (amide II), the difference was not observed between both eyes. We conclude that the difference in diurnal variations of biochemical constituents between right and left eye tear fluids could be monitored noninvasively and nondestructively by FTIR technique and this method could be useful in the future for tear diagnoses.* © 2005 Wiley Periodicals, Inc. Biopolymers 79: 18–27, 2005

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Keywords: *tear fluid; FTIR; noninvasive; diurnal; individual; right and left*

INTRODUCTION

Human ocular surface, as proposed by Thoft[1] in 1977, is a very functional unit composed of conjunctiva, corneal epithelium, and tear fluid to ensure clear and continuous eyesight. Tear fluid contributes greatly to the maintenance of ocular function, for example, taking incident light smoothly, delivering nutrition and hormones to and from ocular tissues, allowing oxygen permeability, removing metabolic

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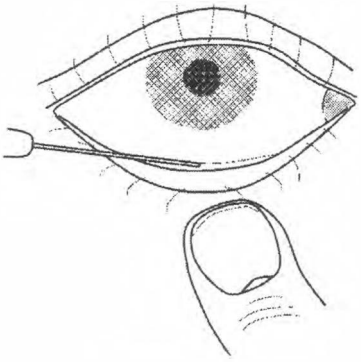
waste, protecting from bacterial attack, and so on.[2-4] It is generally proposed that ocular surface tear fluid has a film-like structural property, which consists of three layers.[5] The superficial layer contains various hydrophobic lipids;[6–9] the intermediate layer is soluble and contains various proteins,[10,11] electrolytes,[12] and other hydrophilic low-molecular-weight compounds such as glucose and urea;[13] and closely on the corneal epithelium is highly viscous layer of mucin.[14,15] These layers do not have distinct borders between each other, but they act as a whole to maintain equilibrium, such as prevention of tear overflow on the lids and evaporation of moisture, by their physicochemical properties.[6,8,16–18]

The components of tear fluid are secreted mainly from the lacrimal gland,[5] the meibomian gland,[7,8] and the goblet cells.[5] It is suggested that they are innervated by the trigeminal, parasympathetic, and sympathetic nerves,[5,19,20] and tear production is under hormonal regulation.[21–23] Taking the idea that biochemical and physiological characteristics of tear fluids may be affected by other organs or tissues, it appears that tear fluid not only plays a significant role in obtaining satisfactory sight, but also is passively affected by physiological changes of human bodies; therefore, tear fluid may tell us valuable information about an individual's health condition.

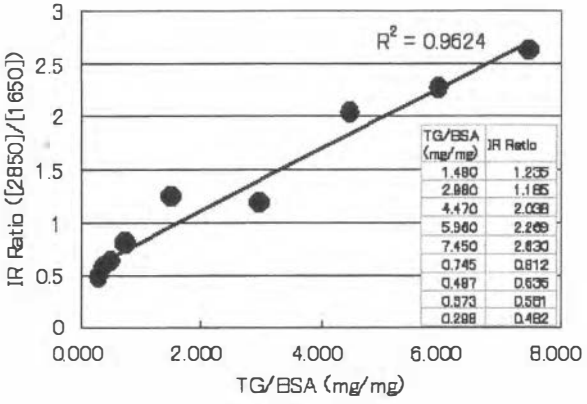
There have been many reports of the analysis of biomolecular components of human body fluids by Fourier transform infrared spectroscopy (FTIR) technique, particularly in the past 10 years. In these studies, for example, human oral mucosa and saliva,[24–28] skin,[29–31] and serum[32–35] were investigated by using FTIR. However, few studies of the analysis of tear fluid by means of FTIR technique exist.

The purpose of this research is to find some useful spectroscopic factors showing diurnal variations in human tear fluid and the difference in those variations between right and left eye tear fluid by utilizing a nondestructive Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR–ATR) method.



Lipid/Protein

(A)



(B)

FIGURE 1 (A) In situ collection method of right eye tear fluid with glass microcapillary tube. (B) Infrared spectral ratio of lipid and protein in a model mixture. The model mixture contains various amounts of triglyceride (triolein) and bovine serum albumin (fatty acid free) in artificial tear solution commercially available (Rohto Pharm. Co., Japan; with 0.5% chondroitin sulfate, 0.1% aminoethylsulfonate in phosphate buffer with KCl, NaCl, and $CaCl_2$ salts). Initially 2.6 mg/mL triolein in ethanol and 1.75 mg/mL bovine serum albumin in phosphate buffer (pH 7.0) were prepared, and 2 μL each was mixed in 40 μL artificial tear solution, followed by preparations of the other samples with increasing amounts of triolein or albumin for changing the lipid-to-protein ratio. In the second derivative spectra of the mixtures, the intensities of peaks for [2850] and [1650] were actually calculated by measuring the difference between 2832 and 2855 cm^{-1} and 1655 and 1690 cm^{-1}, respectively.

MATERIALS AND METHODS

In Situ Tear Fluid Collection

Tear fluid samples were collected from volunteers in Sun Contact lens Co., Ltd. They consisted of one female (subject C, 25 years old) and two males (subject A, 39 years old; subject B, 42 years old). They all awoke between 0600 and 0800 h and had breakfast until 0830 h. Lunch was taken just after the tear collections at 1200 h (subjects B and C) or at 1300 h (subject A), but dinner was not taken until every collection was over. In addition, they worked inside the office and were not burdened physically during tear collections. They had no trouble in their eyes such as inflammation, chronic dry eye disease, or other ocular diseases during and after the experiments. Subjects A and B wear glasses for myopia, but subject C had good sight without them. All subjects do not use contact lenses. Subject A and C have right eye dominance, while subject B has left eye dominance, by their declarations. Informed consent was obtained from each tear fluid donor.

All tear samples were gently taken by using calibrated heat-polished disposable glass microcapillary tubes with

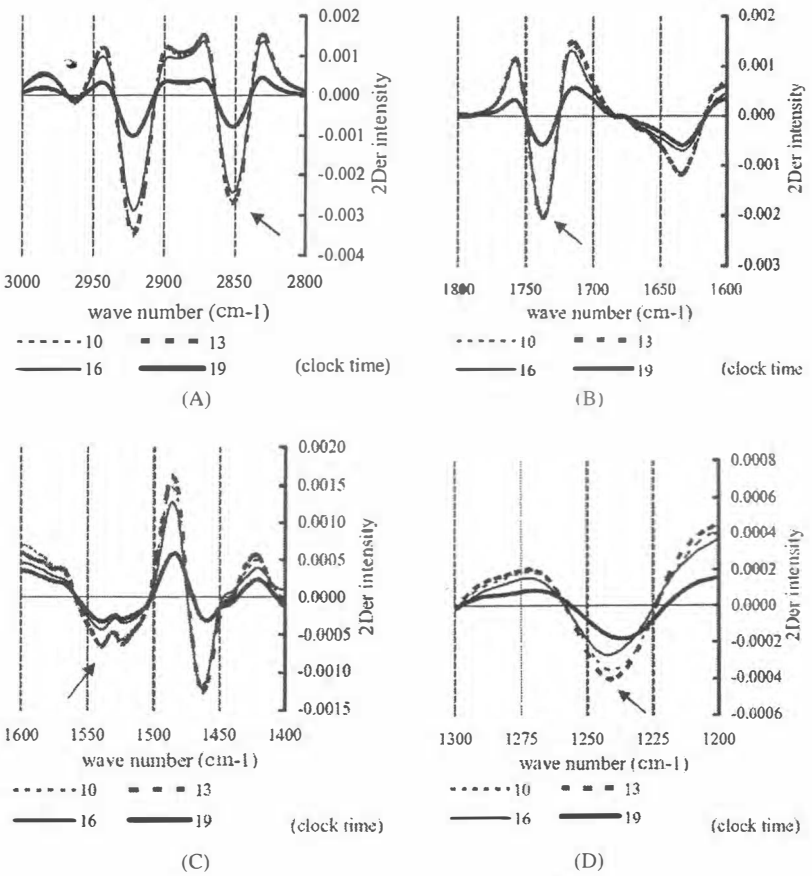


FIGURE 2 The infrared second derivative (2Der) spectra in four peak areas: (A) mainly methylene (CH_2–) band areas between 2800 and 3000 cm^{-1}; (B) mainly carbonyl (C=O) band areas between 1600 and 1800 cm^{-1}; (C) mainly amide II band areas between 1400 and 1600 cm^{-1}; (D) mainly phosphonyl (P=O) band areas between 1200 and 1300 cm^{-1}. In each area, typical four spectra of right eye tear samples at 1000, 1300, 1600, and 1900 h are shown. The arrows in these figures indicate infrared peaks used for calculating the four factors of P/L, P/A, E/L, and E/A.

3 μL capacity from donors' meniscus at the lower lid margin of the each opened eye (Figure 1A). Each tear volume was approximately less than 0.5 μL, normally 0.2–0.4 μL. This method was a modified one reported by Sack et al.[36]

To avoid collecting reflex tear fluid, which is caused by physical stimulation, touching the eyelid margin and corneal surface was strictly avoided. In addition, swift collection (within 10 s at one collection) was also necessary to prevent drying on the ocular surface, which triggers reflex tear fluid. Tear collection was repeated carefully 3 to 12 times at the same hour at 3- to 5-min intervals for each right and left eye; this procedure was carried out three to six times per day with respect to meal time. Tear collection was also performed, taking donors' physical conditions into account, several times in one season irregularly over 2 years.

Treatments of Tear Samples

Tear fluid samples collected by capillary tubes were immediately put directly onto the spot of 1 mm diameter on an aluminum film and dried for 2 h in vacuo. They were stored at 4°C until measurement, which occurred within 48 h.

In this treatment, it was important that the area of tear spots and tear volumes on the aluminum film should be made as small as possible to obtain more precise and reproducible data by FTIR–ATR. As the diameter of tear spot and tear volume on the film become smaller, the averaged coefficient of variation becomes smaller. For example, when the spot diameter and tear volume were $\phi = 1.5$ mm and 0.8 μL, respectively, the average coefficient of variation was 27%, while it was improved to about 5% when they were spotted on $\phi = 1.0$ mm and 0.3 μL. We found in microscopic inspections that tear fluid constituents appeared to be spread unevenly over the aluminum film when they were dried in vacuo. To analyze crude tear fluid more accurately using FTIR–ATR, it was necessary to arrange the diameter of sample spot and tear volumes to be as small as possible. With this technique we confirmed the linearity between the infrared peak ratio and the content concentration ratio in a model sample mixture that was deposited on a aluminum film as described above (Figure 1B).

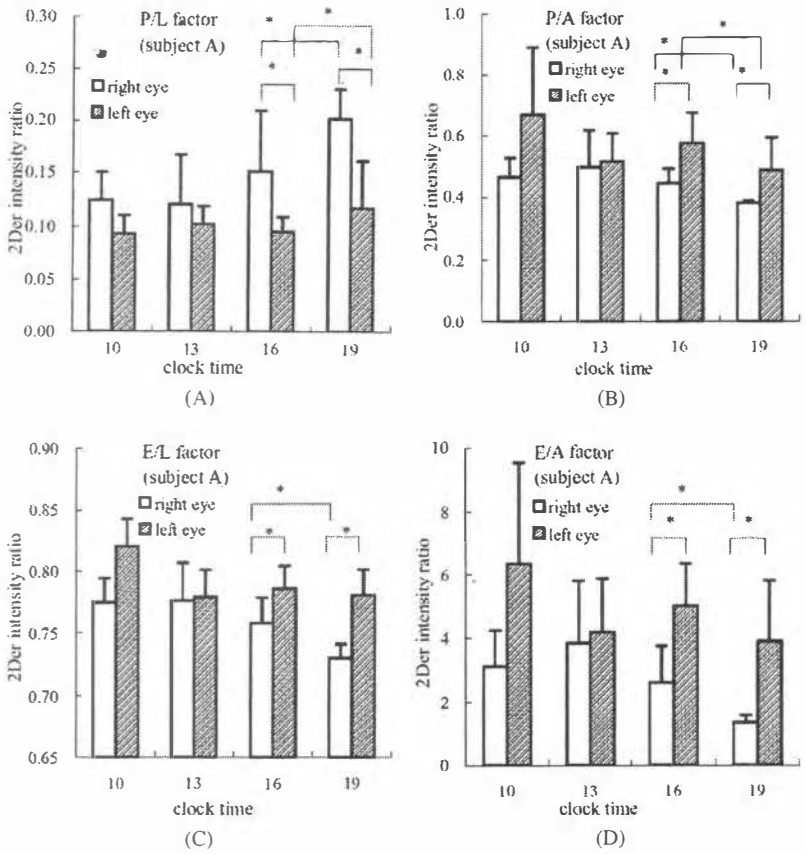


FIGURE 3 The diurnal changes of the four factors, (A) P/L, (B) P/A, (C) E/L, and (D) E/A, compared with right and left eye tear samples obtained from subject A. Averaged infrared second derivative (2Der) spectral intensity ratios (at 1000, 1300, and 1600 h, $n = 51$; and at 1900 h, $n = 15$) are shown. The significant differences from right eye and left eye tear samples at 1600 and 1900 h and the hour-dependent changes from 1600 to 1900 h are demonstrated ($^*P < 0.05$).

Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance

To evaluate a large number of crude tear samples, we used FTIR with a diamond ATR system (TravelIR, SensIR, Columbus, OH). Dried tear sample spot on an aluminum film was placed on the surface of the diamond probe and pressed tightly and then measured at 8 cm^{-1} resolution with 64 accumulations.

Factor Calculations

We used and transformed ir absorption spectral data into the second derivative (2Der) forms (transformed by Savitsky–Golay method with 11 points) to obtain the accurate intensities of the target peaks without the influence of baseline shift and broad bands. We then calculated the peak intensities by measuring the heights between the tops and the troughs of the four main peaks in 2Der spectra and presented the results with the ratios of 2Der intensities. We defined the following four spectroscopic factors (with 2Der intensity at the respective wave number in brackets) that did not depend on tear volumes in the FTIR measurement:

1. Phosphate ester to lipid (fatty acid methylene) ratio (P/L factor): [1242 cm^{-1}]/[2852 cm^{-1}]
2. Phosphate ester to amide (protein amide II) ratio (P/A factor): [1242 cm^{-1}]/[1546 cm^{-1}]
3. Fatty ester to lipid (fatty acid methylene) ratio (E/L factor): [1735 cm^{-1}]/[2852 cm^{-1}]
4. Fatty ester to amide (protein amide II) ratio (E/A factor): [1735 cm^{-1}]/[1546 cm^{-1}]

The data of tear samples collected at the same hour were averaged for evaluation.

Statistical Analyses

Tear samples collected at a fixed time in the same group were analyzed using the Grubs–Smirnov critical test for rejecting outliers in the data. The analyses of intergroup data collected at different times in 1 day were made by one-way layout analysis of variance and Mann–Whitney's U test. Moreover, the right and left eye data obtained simultaneously at a fixed time were analyzed using Welch's t test. In any case, $P < 0.05$ was considered significant.

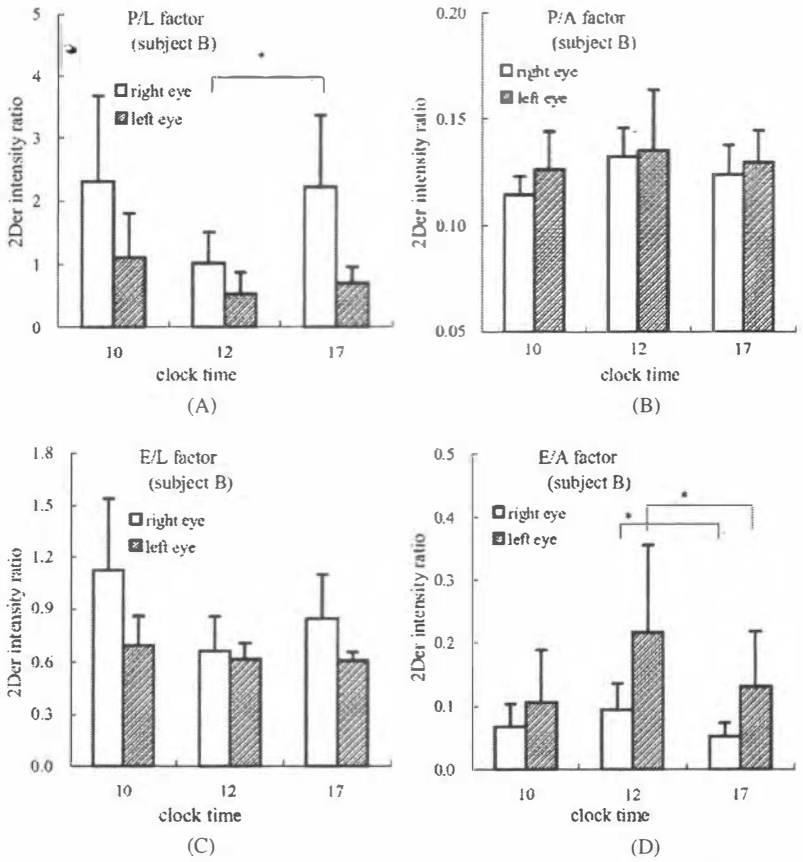


FIGURE 4 The diurnal changes of the four factors, (A) P/L, (B) P/A, (C) E/L, and (D) E/A, compared with right and left eye tear samples obtained from subject B. Averaged infrared second derivative (2Der) spectral intensity ratios (at 1000, 1200, and 1700 h, $n = 3$) are shown. The significant differences from right eye and left eye tear samples at 1600 and 1900 h, and the hour-dependent changes from 1200 to 1700 h are demonstrated ($*P < 0.05$).

RESULTS

Figure 2 shows the typical 2Der forms of the infrared absorption spectra in four notable peak areas for right eye tear samples. The four characteristic peaks at around 2852, 1735, 1546, and 1242 cm^{-1} at 1000, 1300, 1600, and 1900 h were described in the regions of (A) 2800–3000 cm^{-1}, (B) 1600–1800 cm^{-1}, (C) 1400–1600 cm^{-1}, and (D) 1200–1300 cm^{-1}, respectively. In these figures all of the 2Der intensities of those four peaks tended to be lower at 1600 and 1900 h compared to those at 1000 and 1300 h. It was recognized that those four peaks, different from the four factors, lessened in the afternoon than in the morning. We summarized the four factors in Figures 3, 4, and 5.

The intensities of the peaks in 2Der form and the four factor values were ranked in descending order as follows:

[2852 cm^{-1}] > [1735 cm^{-1}] > [1546 cm^{-1}] > [1242 cm^{-1}]

E/A > E/L ~ P/A > P/L

The factor values were variable depending on physiological conditions of the individuals.

Figures 3–5 show the time-dependent changes of the four factors (P/L, P/A, E/L, and E/A), and the results from the three subjects' (A, B, and C) samples are expressed in these figures.

For subject A, the P/L factor of the right eye samples showed increasing tendency from 1600 to 1900 h (Figure 3A), while the left eye samples did not change considerably. Thus, the difference between right and left eye samples for P/L factor in the afternoon (1600 and 1900 h) was significant ($P < 0.05$). In marked contrast to P/L factor, decrements of the other three factors in the right eye were observed, especially E/L and E/A factors, shown in Figure 3C and D, respectively, while a comparatively unchanged profile in the left eye from 1600 to 1900 h was observed. These three factors had similar profiles in this case of subject A and showed significant difference between right and left eyes at 1600 and 1900 h ($P < 0.05$).

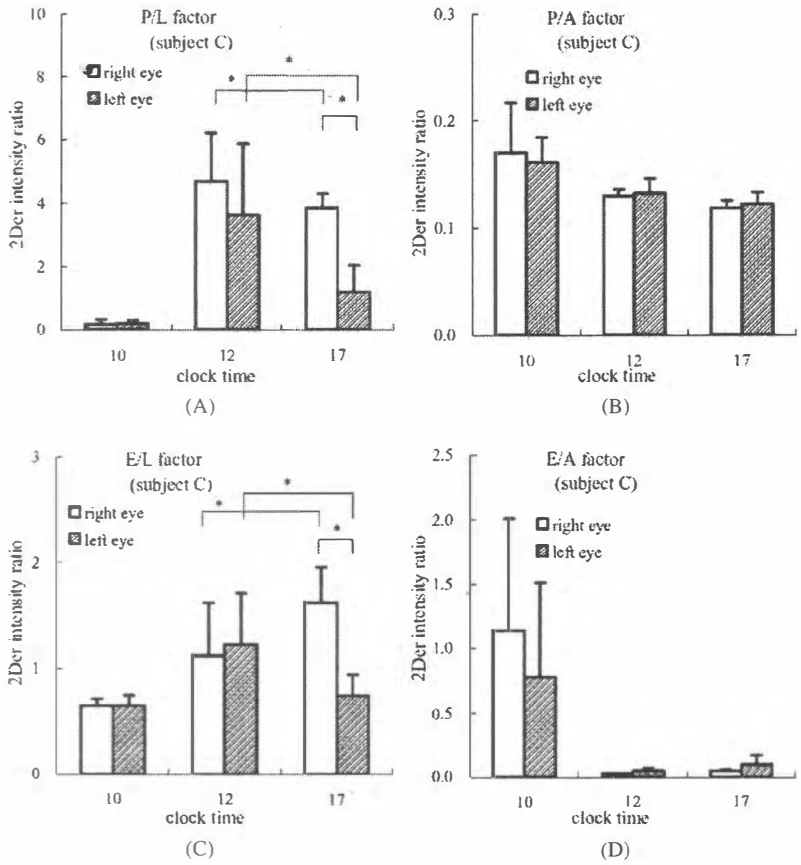


FIGURE 5 The diurnal changes of the four factors, (A) P/L, (B) P/A, (C) E/L, and (D) E/A, compared with right and left eye tear samples obtained from subject C. Averaged infrared second derivative (2Der) spectral intensity ratios (at 1000, 1200, and 1700 h, $n = 3$) are shown. The significant differences from right eye and left eye tear samples at 1600 and 1900 h, and the hour-dependent changes from 1200 to 1700 h are demonstrated ($*P < 0.05$).

For subject B, P/L factor changed similarly to the right and left eyes of subject A from 1200 to 1700 (Figure 4A), while the profiles between 1000 and 1200 (or 1300) h differed. E/A factor shown in Figure 4D represented a different hour-dependent profile from P/L factor, while the profile of E/L factor shown in Figure 4C resembled that of P/L factor.

For subject C, E/A factor represented an opposite relation in the hour-dependent profile to P/L factor as shown in Figure 5D and A, respectively. E/L factor shown in Figure 5C represented a different profile from P/L factor, which differed from subject B.

In Figure 6, the distributions of four factors at 1600 h were shown with horizontal and vertical coordinates for peak intensity pairs of the factors (e.g., 2852 and 1735 cm^{-1} for horizontal and vertical coordinates, respectively). The regression analysis shows that the correlation coefficient between E (1735 cm^{-1}) and L (2852 cm^{-1}) was high ($R^2 = 0.9987$). The correlation coefficient of the other three factors were also high; however, the correlations between P (1242 cm^{-1}) and L (2852 cm^{-1}), and between E (1735 cm^{-1}) and A (1546 cm^{-1}) were not linear. We confirmed that similar profiles of these correlations were also found at other clock times (1000 to 1700 h); this may be a common feature for those four factors. In tear fluid at the same hour, the distribution of the ratio of ester (E: 1735 cm^{-1}) to lipid (fatty acid) (L: 2852 cm^{-1}) was also quite linear.

As shown in Figure 7A, D, we could discriminate the distribution of P/L and E/A plots in tear samples between right and left eyes at 1600 h for subject A. There were significant differences in those factors between right and left eye tear samples ($P < 0.05$). On the other hand, for E/L plots as shown in Figure 7B, identical distribution was observed in both eyes. The distributions for P/A factors as shown in Figure 7C could not be distinguished clearly. These results suggest that there were right and left eye-dependent changes in the intensity variance for those factors.

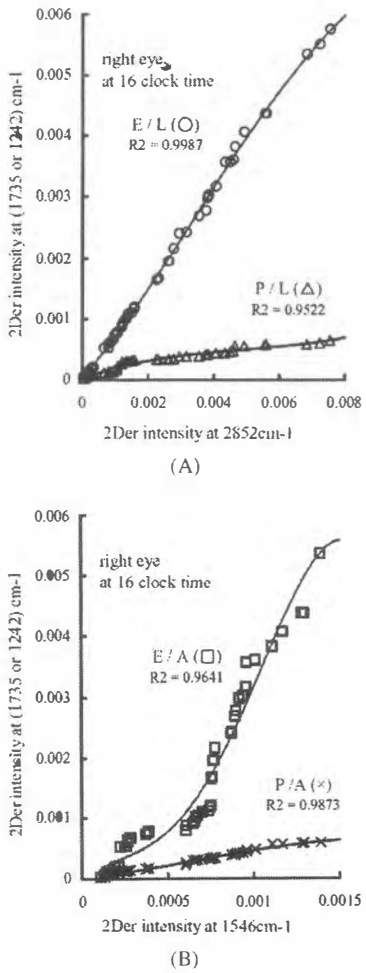


FIGURE 6 The distributions of the four factors, (A) E/L and P/I$_N$ (B) E/A and P/A, expressed in coordinates by both axes of infrared second derivative (2Der) spectral intensity. Right eye tear samples obtained from subject A at 1600 h are shown ($n = 51$). (A) E/L factor represented by circles is plotted with 2Der spectral intensity at around 1735 and 2852 cm^{-1} on the vertical and horizontal axes, respectively. (A) P/L factor represented by triangles uses the same horizontal axis of 2Der spectral intensity at around 2852 cm^{-1} as E/L factor, but on the vertical axis the intensity at around 1242 cm^{-1} is shown. (B) Both E/A and P/A factors represented by squares and crosses are plotted with the intensity at around 1546 cm^{-1} on the common horizontal axis, while on the vertical axis they are plotted with 2Der spectral intensity at around 1735 and 1242 cm^{-1}, respectively.

Figure 8 shows the distinction between subjects A and B (or C) in the distribution of factors E/L (Figure 8A) and P/A (Figure 8B) plots from right eye tear samples at all hours. The difference of factor P/A distribution between subjects A and B, or C, in Figure 8B was significant ($P < 0.05$); however, that difference was not observed in Figure 8A for E/L factor. E/A factor showed a similar profile as P/A factor among these subjects, but P/L factor did not make an obvious distinction between them. These results indicate that the distribution of E/L factor was almost identical for all subjects measured and that the discrimination among those individuals might be clear when using P/A and E/A factors.

DISCUSSION

The diurnal changes of the four spectroscopic factors may be caused by the variations of human tear constituents such as lipids, proteins, and phosphate esters. Since E/L factor (i.e., fatty ester to fatty methylene ratio) did not change much through the daytime and both peak intensities at 1735 and 2852 cm^{-1} may be derived from the almost identical compound, fatty acid ester is a candidate for the spectral origin. Furthermore, as Sack et al.[36] reported previously that the concentration of total tear proteins was 9–16 mg/ml between the states of reflex, open eye, and closed eye. tear fluids contain so many proteins and the amide II peak band at 1546 cm^{-1} may be attributed mainly to tear proteins.

As to phosphate esters, phospholipid and phosphoprotein may be regarded as candidate components of tear fluid. Comparing P/L and P/A factors, the difference of P/L values at 1600 and 1900 h between both eyes was significantly larger and more definite than that of P/A values (Figure 3A, B). A similar relationship is observed in the results of the samples from subjects B and C. On the other hand, P/L factor appears to have an opposite profile to E/A factor. It is suggested that the infrared absorption peak intensity at 1242 cm^{-1} may be derived mainly from phosphoproteins. The contribution from phospholipids to this peak may be little, if present, because the distribution of P/A factor (phosphate ester to protein amide) was nearly linear (Figures 6B and 7C) and that of P/L factor (phosphate to lipid) was not (Figures 6A and 7A), suggesting better correlation of phosphate ester to protein than to lipid.

Tear fluids play an important function as a barrier in the defense mechanism of the eye. The chemical difference between right and left eye tear fluids may cause different effects on the homeostasis of each ocular surface. For contact lens wearers, it is necessary to be make a distinction between the ocular surface conditions of both eyes, because the contact lenses, with tear fluids, largely disorder the physiological balance of defense mechanisms on the ocular surface. The chemical constituents such as proteins in tear fluids interact with the materials of lenses and

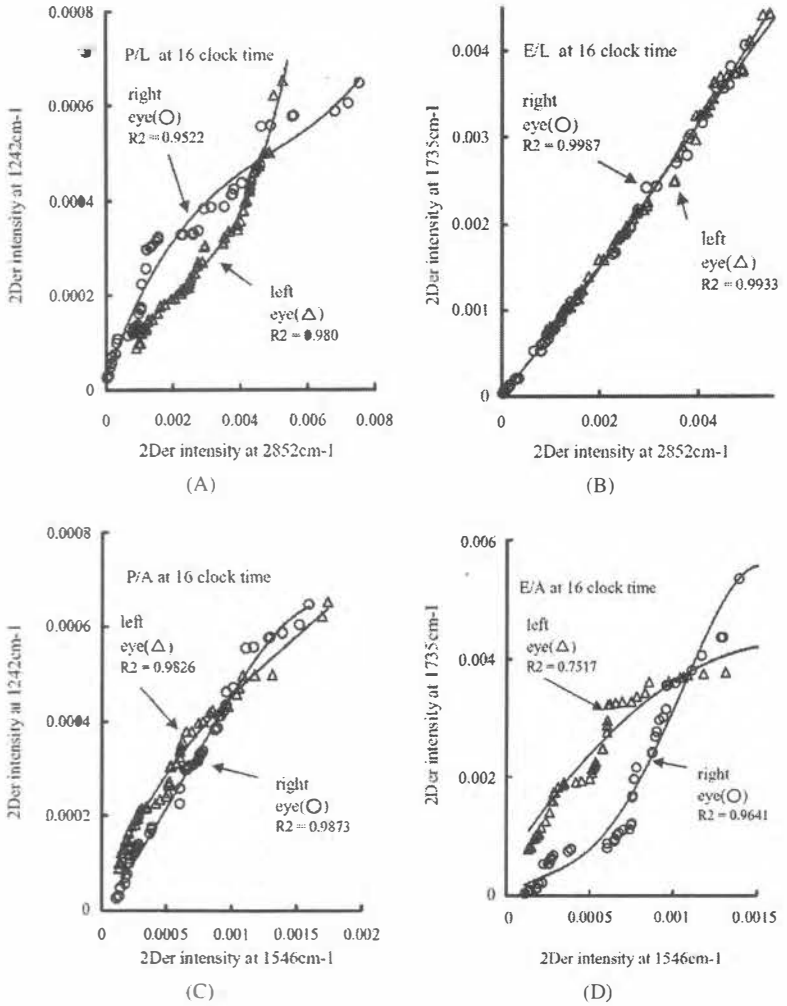


FIGURE 7 The distributions of right and left eye tear samples of (A) P/L, (B) E/L, (C) P/A, and (D) E/A factors obtained from subject A, expressed in coordinates by both axes of infrared second derivative (2Der) spectral intensity. Right (○) and left (△) eye tear samples at 1600 h are shown ($n = 51$). Each pair of factors with 2Der spectral intensity are plotted on the vertical and horizontal axis.

adhere to the plastic surfaces of lenses, causing contamination and deterioration, especially in the case of hydrogel lenses.[37–39] It is suggested that this deposition on lenses is a scaffold for bacteria and may cause inflammatory or infectious diseases on the ocular surface.[40] The smooth movement of hard contact lenses by blinking in tear fluids may be altered by the changes of tear film structure and fluidity, resulting in poor fit and risky wear. In any case, the quality of tear fluids has a great influence on contact lenses, and it is important to investigate the interaction between the constituents of tear fluid and the material or shape of the contact lens to avoid ocular surface disorders.

It may be also useful to analyze the quality of tear fluids for identifying and preventing ocular diseases. In dry eye syndrome the typical patient shows a reduced tear volume and rapid tear break-up time (the time for dry spots to occur on the cornea).[41] This happens from an imbalance between tear production and drainage, but its mechanism is not clear. It usually shows the structural change of tear film,[42] which has some deficits in the three layers in addition to reduced tear volume. The variation of tear constituents may reflect the stability of ocular surface tear film, and, moreover, the effect of body condition on the ocular surface may be predicted in the near future by the analyses of diurnal or nocturnal changes of tear fluid constituents.

Glaucoma is a disease that damages the optic nerve and consequently causes visual loss. A glaucoma patient usually shows a high intraocular pressure, but some patients have pressure in the normal

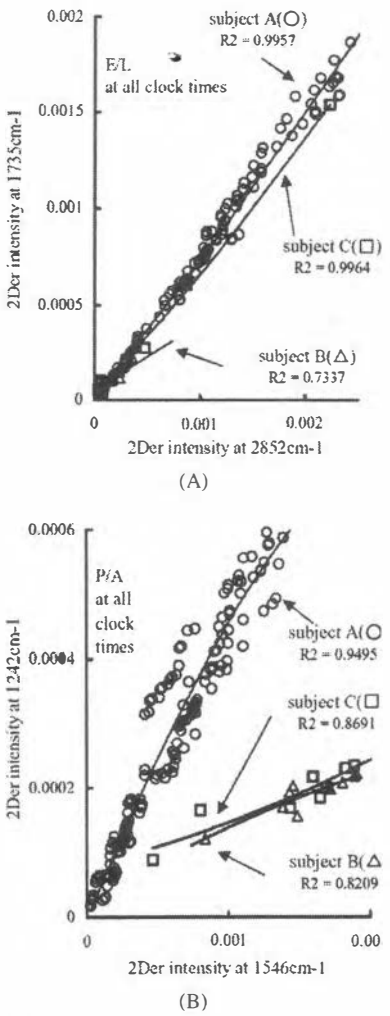


FIGURE 8 The distributions of (A) E/L factor and (B) P/A factor obtained from subjects A (○), B (△), and C (□), expressed in coordinates by both axes of infrared second derivative (2Der) spectral intensity. Right eye tear samples at all hours measured are shown (subject A, $n = 168$; subjects B and C, $n = 9$). Each pair of factors with 2Der spectral intensity are plotted on the vertical and horizontal axis. There is no clear distinction in E/L factors among the three subjects, but obvious discrimination has been observed in P/A factors between subjects A and B or C.

range. It has been reported that intraocular pressure shows diurnal fluctuation[43–45] and a difference between right and left eyes. It could be important to reveal the relationship between changes of tear fluid constituents and intraocular pressure for elucidating the pathophysiology of glaucoma.

In this study no external injuries or diseases on the ocular surface were observed nor claimed by any subjects. It may be reasonable to think that the difference between right and left eye tear fluids could be caused by substantial reasons of morphogenetic or neuropathological differences on each eye.

Ocular dominance[46–48] is one of the most noticeable aspects for the distinction between physiological states of both eyes. It may affect steadily the growth and development of ocular tissues, with different sight and sensing of external subjects and conditions. Ocular dominance may produce the effect with physiological reactions to the visual cortex in the central nervous system.

At present we cannot show that each dominant eye of three subjects corresponded to the diurnal changes of P/L factor. The mechanism of diurnal changes of the spectral factors might be too complicated to explain the physiological difference only by eye dominance, because the physiological difference of both tear fluids may be affected by other organic conditions concerned with right- and left-oriented mechanisms of total bodies. We should consider at least the eyesight, intraocular pressure, right- or left-handedness, and some genetic factors. Moreover, we need further analyses to identify the infrared peak compounds showing the diurnal changes in tear fluids of both eyes by chemical methods.

CONCLUSION

We conclude that the differences in diurnal variation of biomolecular constituents in right and left eye tear fluids can be monitored qualitatively and nondestructively by FTIR technique.

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