FTIR ANALYSIS OF TEAR FILM CHANGES ASSOCIATED WITH DRY EYE SYNDROME AND POTENTIAL TREATMENT WITH TOPICALLY APPLIED OLIVE OIL AND FLAX SEED OIL

*A. KHALIL**, MERVAT A. ALY*, S.M. SHERIF*[#], MONA S. TAALAT**, A.M. SALLAM***

* Biophysics and Laser Science Unit, Research Institute of Ophthalmology, Giza, Egypt,
[#]e-mail: sheri_sm@yahoo.com
** Biophysics Branch, Faculty of Science, "Ain Shams" University, Abbasia, Cairo

Abstract. Dry eye is a multifactorial condition that affects the surface of the eye and induces an inflammatory response that affects the cornea. Recent studies have shown beneficial effects of dietary supplementation and/or topical application of olive oil and flax seed oil containing omega-3 and omega-6 polyunsaturated fatty acids, for dry eye condition treatment. In this study, the effects of the induced dry eye condition and the topical application of olive oil and flax seed oil on the tear film structure was monitored after 4 and 8 days of application. The tear film analysis was accomplished by the application of Fourier transform infrared spectroscopy (FTIR). The results demonstrated that dry eye, induced by 0.1% benzalkonium chloride (BK), was found to induce changes in the tear film structure (fatty acid and protein components) through destruction and formation of new hydrogen bond that specially appeared in the NH, OH, amide I and fingerprint regions, and the topical uses of olive oil and flax seed oil for 8 days have reversed these effects on tear film constituents.

Key words: Dry eye, olive oil, flax seed oil, 0.1% BK, FTIR.

INTRODUCTION

Dry eye syndrome is a common external eye disease that arises from a wide variety of etiologies. The prevalence of dry eye has been reported as 9% of patients over 40 years of age, increasing to 15% of those over 65 [7, 11]. Dryness, redness, foreign body sensation, burning and itching of the eyes are typical symptoms of dry eye [2]. Somewhat controversially, intermittent excessive tearing can also be the main symptom of irritated eye. These symptoms are often unpleasant; moderate cases may affect quality of sight and life, but severe cases may lead to the damage of the ocular surface, resulting in impaired vision or even perforation of the eye [6]. Naturally occurring essential fatty acids – omega-3 and omega-6 series – found in olive oil and flax seed oil are promising natural anti-inflammatory agents shown to

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have beneficial effects in many inflammatory conditions such as rheumatoid arthritis, ulcerative colitis and also ameliorated the signs and symptoms of dry eye [4]. Recent studies have shown that topical application of omega-3 and omega-6 fatty acids may be a novel therapy to treat the clinical signs and inflammatory changes accompanying dry eye syndrome [10].

The tear film has a three-layered structure consisting of an anterior lipid layer, a middle aqueous layer (mainly water and protein) and a deeper mucin layer [15]. Aqueous tear deficiency and increased evaporation may contribute to the dry eye condition. In this regard, any changes in the protein – lipid structure of the tear film will be one of the main effects of dry eye [19].

The aim of this work is to investigate the effect(s) of dry eye syndrome on the molecular constituents of the tear film and the possibility of using the natural oils (olive oil and flax seed oil) as treatment modalities.

MATERIALS AND METHODS

MATERIALS

Commercially available benzalkonium chloride (BK) – a preservative in drug industry – was obtained from Alpha Chemika, Mumbai, India. Extra virgin olive oil and flax seed oil were obtained from the local market and were produced by the use of physical means and no chemical treatment.

Twenty eight healthy mature Rex (colored) rabbits of both sexes with age 4–5 months and weighing 2–2.5 kg were randomly selected from the animal house facility at the Research Institute of Ophthalmology, Giza, Egypt. The animals were housed in specially designed cages and maintained under constant air flow and illumination during the experimental periods. They were supplied with standard diet and water ad libitum. Animals were divided into four groups. Each group composed of seven animals of both sexes. Group A served as the control one. Groups B, C and D were used to induce the dry eye model as given below, then groups C and D were treated with either olive oil or flax seed oil, respectively. Animals were handled according to the ARVO (The Association for Research in Vision and Ophthalmology) statements for the use of animals in research, and the research protocol was approved by the local ethical committee.

INDUCTION OF DRY EYE AND TREATMENT REGIMEN

Induction of dry eye condition was previously described [16]. Briefly, benzalkonium chloride (0.1%) was twice-daily topically administered in both eyes of groups B, C and D for 10 days. After 24 hours of inducing the dry eye, olive oil and flax seed oil, without dilution, were topically administered into both eyes of the rabbits in groups C and D, twice daily, respectively, and the possible effects were evaluated after 4 and 8 days of application.

TEARS COLLECTION AND FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

In the morning, fresh tears were obtained from rabbit eyes of all groups using a micropipette. Collection of tears was performed after four and eight days of treatment with the natural remedies, i.e. olive oil and flax seed oil. The collected tears were individually freeze-dried and kept under N_2 gas atmosphere for the infrared spectroscopic analysis.

For the spectroscopic investigation, the KBr pellets were ground in a mortar to produce the fine powder suitable for preparing the KBr-disks that will be used in analysis. Then the dried tears residues were added, and the mixture was mixed. The resulted mixture was placed in a special holder provided by the manufacturer and pressed under vacuum for 2 min to produce the IR–KBr disks. FTIR measurements were carried out using Nicolet-iS5 infrared spectrometer (ThermoFisher Scientific Inc, Madison,USA) with effective resolution of 2 cm^{-1}. Each spectrum was derived from 100 sample interferograms. The spectrometer was operated under a continuous dry N_2 gas purge to remove interference from atmospheric CO_2 and H_2O vapor. The spectra were baseline corrected, then smoothed with Savitsky–Golay filter to remove the noise before Fourier transformation. Three spectra from each sample were obtained and averaged using OriginPro8 software (Origin Lab Corporation, Northampton, MA, USA) to obtain the final average group spectrum which was normalized according to certain peaks and used in the figures.

STATISTICAL ANALYSIS

Data were expressed as the mean ± SD. Comparison between multiple groups was performed using analysis of variance (ANOVA); commercially available statistical software package (SPSS-11 for Windows) was used where the significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

The FTIR spectra of tears for control, 0.1% BK (dry eye model), olive oil and flax seed oil groups covering the range 4000–1000 cm^{-1} were shown in Figure 1. The detailed spectral analyses were performed in four distinct frequency ranges; 4000–3000 cm^{-1} (NH, OH region), 3000–2800 cm^{-1} (CH stretching region), 1800–900 cm^{-1} (fingerprint region) and 1700–1600 cm^{-1} (amide I region).

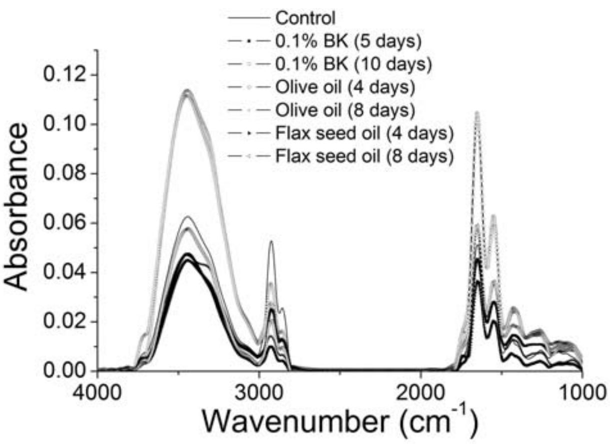

Fig. 1. Overlaid FTIR spectra for all studied groups.

NH,OH REGION

Fig. 2 shows the spectra of the NH,OH region of the protein part of the tear film [19]. The control pattern indicates the presence of the main broad band at 3458±2 cm^{-1}. After deconvolution procedure this main band was resolved into four bands, at 3598±2, 3477±2, 3294±1 and 3078±2 cm^{-1}, that correspond to stretching OH ($_{str}OH$), stretching OH asymmetric ($_{str}OH_{asym}$), stretching OH symmetric ($_{str}OH_{sym}$) and CH ring (CH_{ring}) respectively [2].

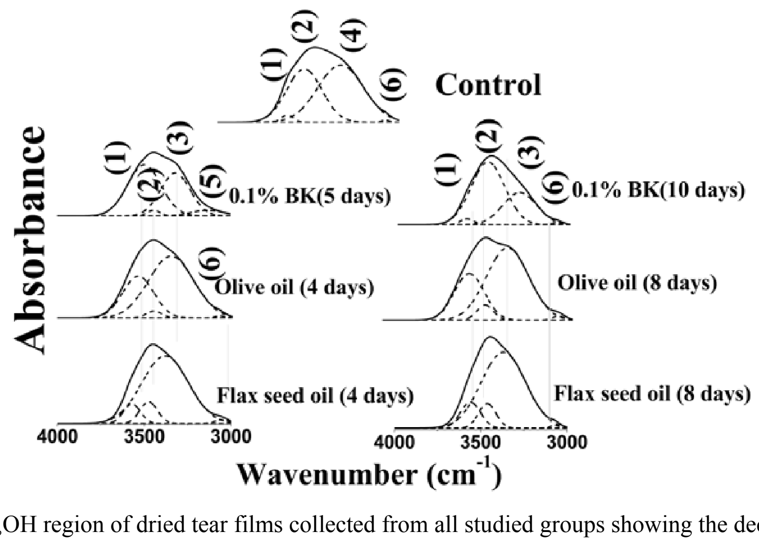

Fig. 2. NH,OH region of dried tear films collected from all studied groups showing the deconvoluted FTIR spectrum. 1 – $_{str}OH$, 2 – $_{str}OH_{asym}$, 3 – $_{str}NH_{asym}$, 4 – $_{str}OH_{sym}$, 5 – $_{str}NH_{sym}$, 6 – CH_{ring}.

In association with induction of dry eye, there are detectable changes in the tear film structure which appeared in the NH,OH region. After 5 days of exposing the rabbit eyes to 0.1% BK, the CH_{ring} and $_{str}OH_{sym}$ vibrational modes were restricted while the $_{str}NH_{sym}$ was detected; this band can be used as a marker for dry eye diagnosis. There is shift in the wavenumber of the $_{str}OH$ and $_{str}OH_{asym}$ mode bands toward lower one, associated with changes in bandwidth, which indicates the formation of hydrogen bonds with different structural states. After 10 days of exposure to 0.1% BK, the $_{str}OH_{sym}$ vibrational mode was also restricted. It is also noticeable that there are shiftings of the $_{str}OH$ and $_{str}OH_{asym}$ mode bands toward a higher wavenumber, with changes in bandwidth, which indicates that hydrogen bonds have been destructed and/or weakend [17].

After treatment with olive oil, there is shifting of $_{str}OH$ and $_{str}OH_{asym}$ mode bands toward a lower wavenumber, with changes in bandwidth, which indicates that the olive oil has reversed the effect of the 0.1% BK on these bands. After treatment with flax seed oil, the $_{str}OH$, $_{str}OH_{asym}$ and CH_{ring} mode bands were mimicking the control with decrease in bandwidth in $_{str}OH_{asym}$ only.

The above findings indicate that there are changes in the protein structure of the tear film associated with dry eye and both olive oil and flax seed oil have protective effects on the tear film. These findings were supported by the findings of Saadia *et al*. [10], He *et al.* [5] and Rand *et al.* [9].

Table 1

Estimated structural components and their vibrational frequencies of the NH, OH region

	Control	**0.1% BK**		**Olive oil**		**Flax seed oil**	
		5 Days	**10 Days**	**4 Days**	**8 Days**	**4 Days**	**8 Days**
$_{str}$**OH**	3598±2 75±3	†3497±3 †193±2	†3615±2 †64±3	†3538±2 †177±8	†3543±3 †156±2	†3559±3 †117±2	3593±3 78±3
$_{str}$**OH**$_{asym}$	3471±2 194±2	†3442±2 †74±1	†3522±1 195±2	†3447±2 †89±9	3468±3 †88±6	†3465±3 †102±9	3470±3 †101±6
$_{str}$**NH**$_{asym}$		†3317±2 †171±1	†3322±2 †253±1	†3350±3 †256±5	†3342±2 †222±3	†3368±3 †250±9	†3379±3 †112±4
$_{str}$**OH**$_{sym}$	3294±1 193±4						
$_{str}$**NH**$_{sym}$		†3132±2 †189±1					
CH$_{ring}$	3067±3 60±3		†3075±2 †46±2	3072±3 54±6	3073±3 63±3	3069±3 54±6	3065±3 65±6

First line in each cell indicates the vibrational frequency, while second line reflects the bandwidth.
†Statistically significant.

CH REGION

Fig. 3 shows the vibrational frequency range 3000–2800 cm^{-1} that corresponds to the CH stretching region of the lipid part of the tear film [14]. The control pattern indicates the presence of three bands in the pattern and the curve enhancement procedure also confirms the presence of these bands that were centered at 2964±3, 2924±2 and 2856±2 cm^{-1} and can be assigned to $CH_{3\ asym}$, $CH_{2\ asym}$ and $CH_{2\ sym}$ stretching vibrations respectively.

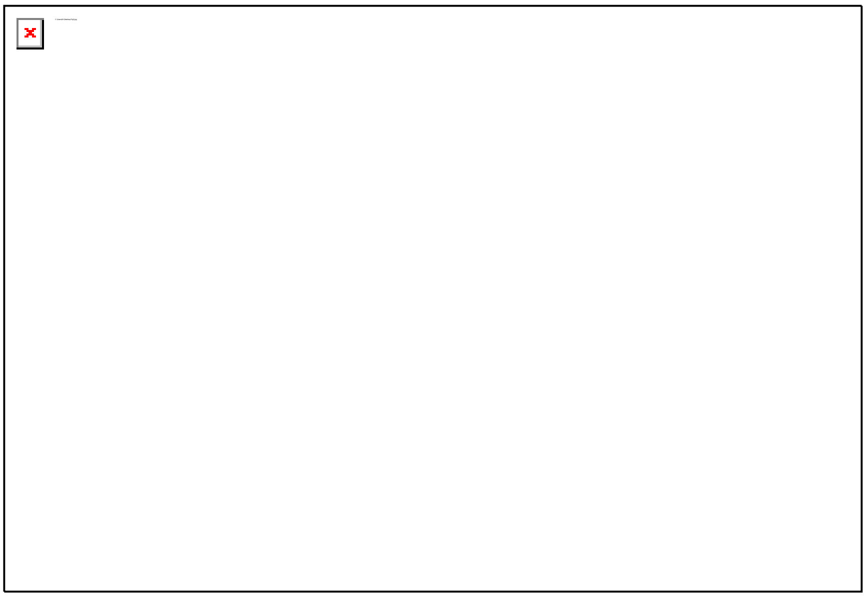

Fig. 3 CH region of dried tears collected from all studied groups showing the deconvoluted FTIR spectrum. 1 – $_{asym}CH_3$, 2 – $_{asym}CH_2$, 3 – $_{sym}CH_2$.

As shown in Table 2, there were no significant changes in the number of deconvoluted bands as well as in their wavenumber or the bandwidth, this data indicate that the CH region of tears-lipids is not affected by 0.1% BK, and both olive oil and flax seed oil had no structural or conformational effects on this stretching region.

Table 2

Estimated structural components with their vibrational frequencies of CH region

	Control	**0.1% BK**		**Olive oil**		**Flax seed oil**	
		Day 5	**Day 10**	**Day 4**	**Day 8**	**Day 4**	**Day 8**
$CH_{3\ asym}$	2964±2 27±1	2964±2 25±3	2963±1 26±2	2962±2 27±1	2962±2 29±2	2964±1 24±3	2964±1 25±2
CH_{2asym}	2924±2 39±3	2922±2 40±1	2921±2 36±3	2920±3 36±3	2920±3 34±5	2924±1 38±2	2922±2 39±1
$CH_{2\ sym}$	2856±2 29±2	2855±2 31±3	2853±1 27±2	2854±2 30±1	2853±2 29±2	2853±3 29±1	2853±3 30±1

First line in each cell indicates the vibrational frequency, while second line reflects the bandwidth.

FINGERPRINT REGION

Fig. 4 shows the vibrational frequency range 1700–900 cm^{-1} corresponding to the fingerprint region that presents the lipid and protein parts of the tear film [12]. The control pattern was characterized by seven bands and the curve enhancement procedure also confirms the presence of these bands that were centered at 1455±3, 1413±2, 1305±3, 1249±2, 1160±3, 1118±3 and 1075±2 cm^{-1} and correspond to CH_2 bending, $_{sym}COO$, CH_3 bending, $_{asym}PO_2$, $_{asym}COOC$, $_{asym}POC$ and $_{sym}PO_2$ respectively [3].

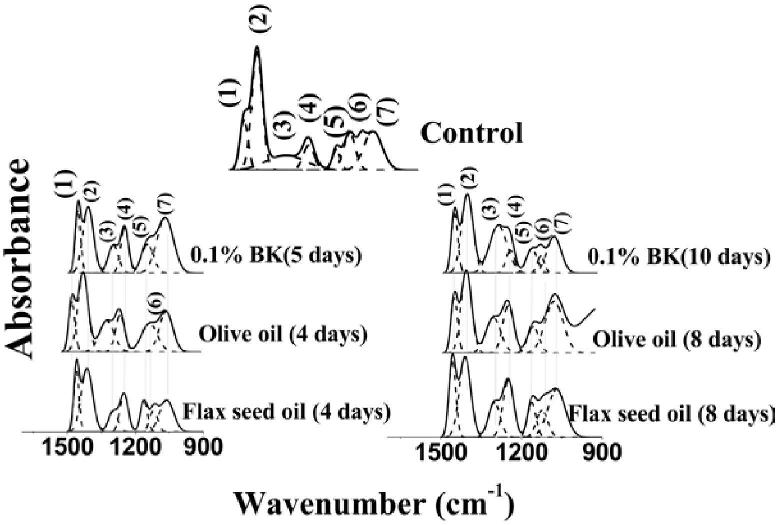

Fig. 4. Fingerprint region of all studied groups showing the deconvoluted FTIR spectrum. 1 – CH_2 bending, 2 – $_{sym}COO$, 3 – CH_3 bending, 4 – $_{asym}PO_2$, 5 – $_{asym}COOC$, 6 – $_{asym}POC$, 7 – $_{sym}PO_2$.

In association with induction of dry eye, the $_{asym}POC$ vibrational mode was restricted after 5 days of exposing to 0.1% BK and then formulated after 10 days. In addition, there are changes in the lipid and protein molecular motions which are confirmed by broadening of $_{sym}COO$ mode band (mainly from fatty acid) after 5 days of induction eye dryness. There is shifting of the CH_{3bend} and $_{asym}COOC$ (mainly from protein) toward lower wavenumber after 10 days of induced eye dryness. These results indicate that dry eye caused changes in the fatty acid and protein structure of the tear film.

After four days of treatment with olive oil, the $_{asym}COOC$ vibrational mode band was absent while $_{asym}POC$ mode band was undetected after eight days of treatment. In the same context, the other bands were mimicking the control pattern. After treatment by flax seed oil, the bands were characterized by wavenumbers and bandwidths that mimic the control spectra and flax seed oil was found to induce refinements in the CH_3 bending, $_{asym}PO_2$, $_{asym}COOC$ and $_{sym}$ PO_2 mode bands.

Table 3

Estimated structural components and their vibrational frequencies in the fingerprint region

	Control	0.1% BK		Olive oil		Flax seed oil	
		Day 5	Day 10	Day 4	Day 8	Day 4	Day 8
CH_2 bending	1455±2 30±2	1458±2 28±2	1458±3 29±2	1455±2 31±1	1456±1 30±2	1459±3 27±4	1456±1 30±2
$_{sym}$COO	1413±2 40±6	1412±2 †54±3	1409±3 51±6	1407±2 50±4	1409±3 48±6	1413±2 50±5	1410±3 49±5
CH_3 bending	1304±2 95±5	1300±3 †52±7	†1287±2 †79±3	1306±1 †73±8	1302±3 86±7	1299±3 †48±2	1303±3 †50±2
$_{asym}PO_2$	1249±3 41±5	1250±2 40±2	†1240±3 39±2	1248±2 45±4	1243±3 51±9	1248±1 42±1	1248±1 46±5
$_{asym}$COOC	1160±2 29±2	†1148±3 †62±2	†1152±2 †48±8		†1148±2 †52±7	1161±2 33±2	1161±1 36±7
$_{asym}$POC	1118±2 44±2		1121±3 32±8	1122±4 79±9		1118±1 41±3	1123±3 49±5
$_{sym}$ PO_2	1075±3 69±2	1070±2 †85±7	†1064±2 64±5	†1059±8 67±2	1070±3 †83±9	1069±3 71±2	1073±2 76±6

First line in each cell indicates the vibrational frequency, while second line reflects the bandwidth.
† Statistically significant.

AMIDE I BAND

The other frequency range under consideration is 1700–1600 cm^{-1}, which is shown in Fig. 5. The main peak of the control amide I band was centered at 1654 cm^{-1}. The curve enhancement procedure resolved the contour of the control band into 3 compositional bands that were centered at 1681 cm^{-1} (β-turns), 1657 cm^{-1} (α- helix) and 1629 cm^{-1} (β-sheet) [17]. It is also noticeable that there were no significant changes in the number of the estimated bands as well as in their wavenumber or the bandwidth in all groups. Table 4 indicates that the distribution of control protein secondary structure components that were calculated as the area percentage were 71.2±3% for α-helix, 19.7±1.4% for β-sheet, and 9.1±1% for β-turns.

Several observations can be made based on the data in Table 4. Firstly, there was a decrease in protein solubility in association with induction of dry eye due to the decrease in α-helix content and increase in β-sheet content. It was reported that the increased β-sheet content resulted in the more insoluble protein [18]. Secondly, after treatment by olive oil and flax seed oil, the protein became more soluble due to increase in α-helix and decrease in β-sheet contents. Thirdly, the β-turns were increased in association with induction of dry eye while it turned similar to control after treatment with olive oil and flax seed oil. Fourthly, the detected β-sheet in all groups with frequency >1620 cm^{-1} are assigned as intermolecular β-sheets [13]. Finally, our results indicate that dry eye caused changes in the protein secondary structure of the tear film and these changes were reversed after treatment with olive oil and flax seed oil, also confirmed by amide I region results.

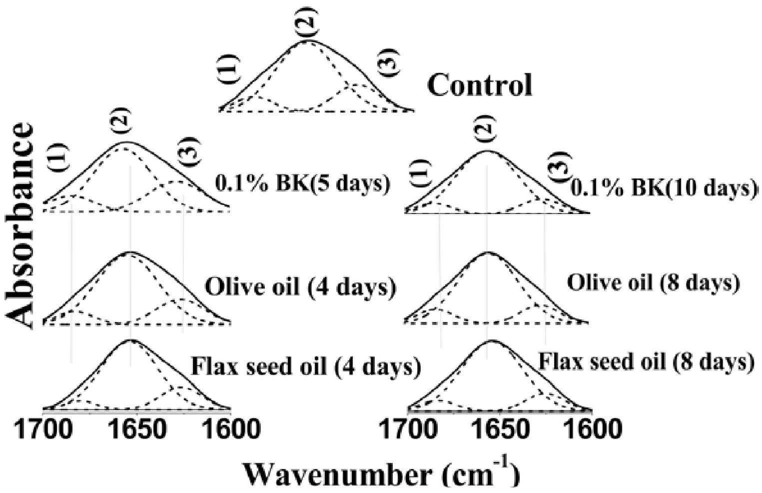

Fig. 5. Amide I region of the tear's protein showing the protein secondary structure components after deconvolution. 1 – β-turns, 2 – α-helix, 3 – β-sheet.

Table 4

Protein secondary structure of tears collected from all studied groups, expressed as area percentage of each structural component relative to the total band area

	Control	0.1% BK		Olive oil		Flax seed oil	
		Day 5	Day 10	Day 4	Day 8	Day 4	Day 8
β-sheet	19.7±1.4	†12.4±1	†27.6±2	20.1±1.5	†13.8±1	18.5±1.5	†15±1.5
α-helix	71.2±3	†79.8±2	†60.5±2	72±2	77±3	75±4	76.5±4
β-turns	9.1±1	7.8±0.7	†11.9±0.5	7.9±0.8	9.2±1	†6.5±1	8.5±1

† Statistically significant.

CONCLUSIONS

Dry eye condition was associated with changes on the tear film structure which appears after exposure to 0.1% BK for 5 to 10 days. These changes clearly appeared in the NH, OH, amide I and fingerprint regions. The topical uses of olive oil and flax seed oil in case of dry eye were associated with different beneficial effects on the tear film constituents, as shown by the changes toward control of the same FTIR bands. In summary, olive oil and flax seed oil can be considered as a novel treatment modality for dry eye syndrome and further studies are required to optimize dosing and formulations that are maximally effective.

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