Fluorophores of lipofuscin granules responsible for human eye fundus autofluorescence

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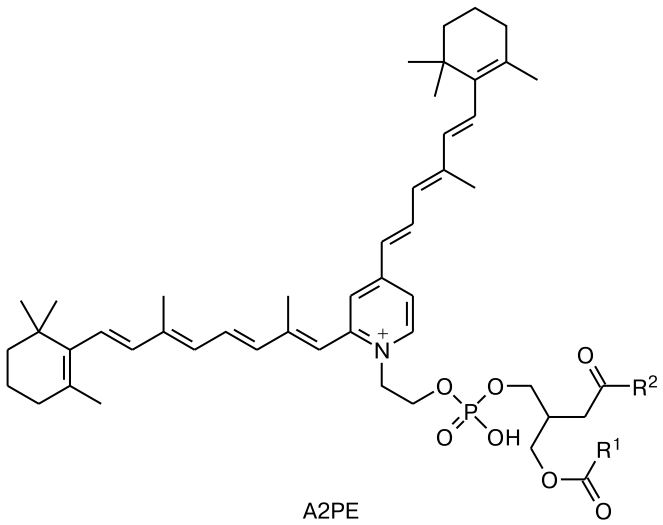
Individual fluorophores and/or their groups contained in a chloroform extract of lipofuscin granules isolated from retinal pigment epithelium of human cadaver eyes were studied by HPLC. Their spectral characteristics were studied, which made it possible to evaluate the contribution of particular fluorophores and/or their groups to the general image of human eye fundus autofluorescence. Many components, being conjugates of *all-trans*-retinal of different nature, contribute to the total fluorescence spectrum of the chloroform extract. The fluorophore A2E is not predominant.

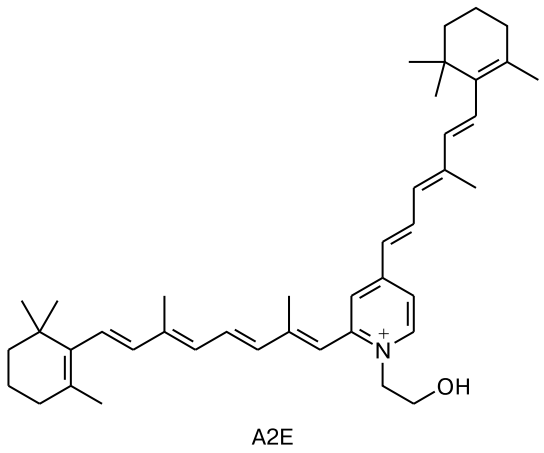
Key words: retinal pigment epithelium, lipofuscin granules, fluorophores, human fundus autofluorescence, retina degeneration, fluorescence, medical chemistry.

A new non-invasive diagnostic technique of senile changes and degenerative diseases of human eye retina and retinal pigment epithelium (RPE) is based on the detection of fluorescence of lipofuscin granules (LG) or "old age pigment", which is located in RPE cells.[1] This method makes it possible to estimate the degree of safety and viability of the RPE/retina complex in various visual pathologies. The recent studies revealed a correlation between the accumulation of LG in RPE cells and such degenerative diseases of retina as age macular degeneration, Stargardt´s disease, and several others.[2] Particles of LG are formation of lipid—protein nature containing up to 10—12 fluorophores[3] and, hence, they can strongly fluoresce. Another important property of LG is their ability to photogenerate active forms of oxygen[4] because of which they are phototoxic.

Fluorophores of LG are mainly conjugates of *all-trans*-retinal (ATR).[5—7] The first of them was identified and characterized as *N*-retinyl-*N*-retinylidene ethanolamine (A2E),[8] which is the product of interaction of two ATR molecules with the amino group of one of the lipids of the photoreceptor membrane, namely, phosphatidylethanol-



A2PE



A2E

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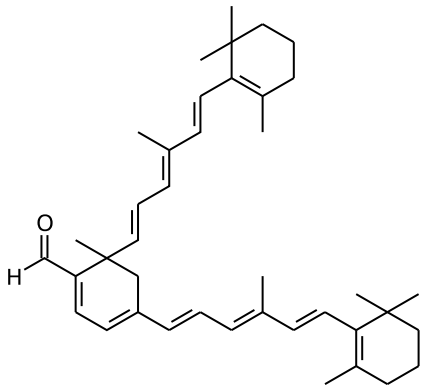
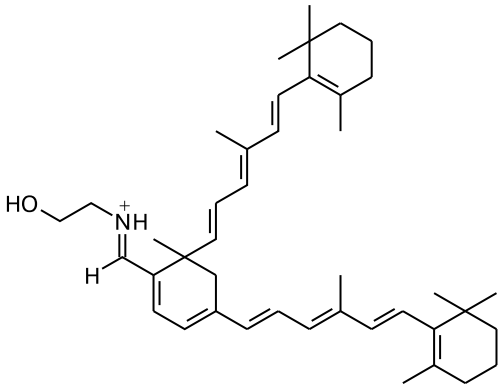
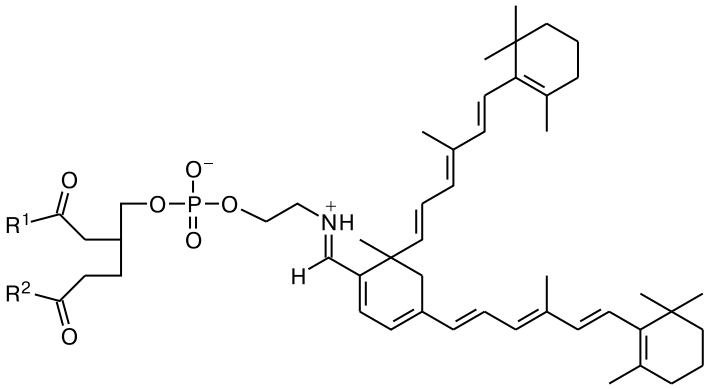


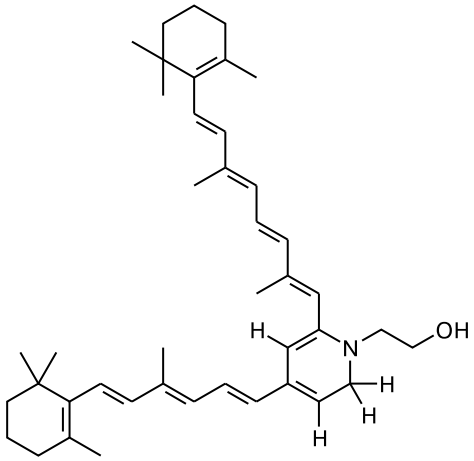
ATR-dimer



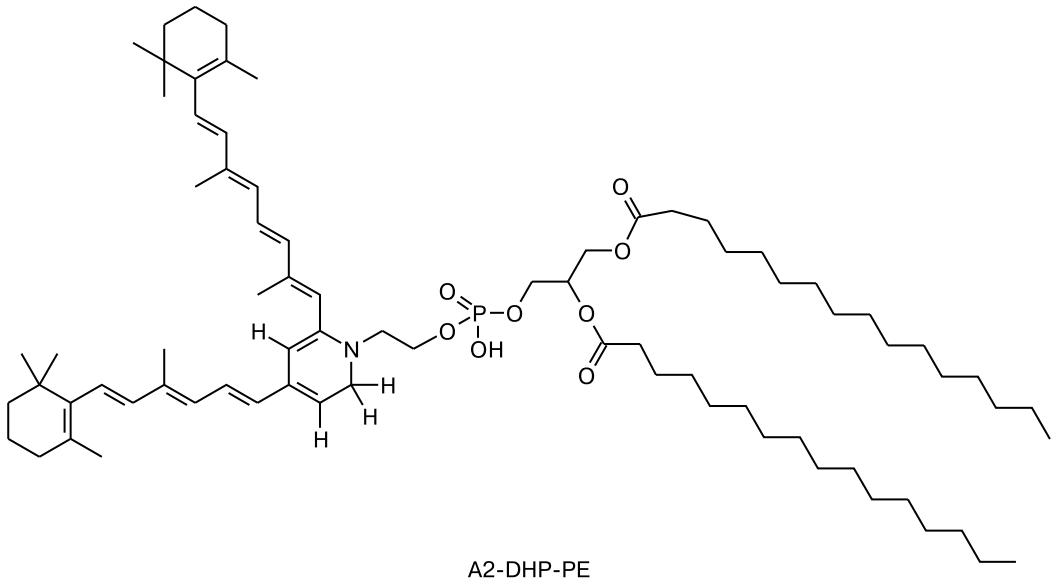
ATR-dimer-E



ATR-dimer-PE



A2-DHP-E



A2-DHP-PE

amine (PE). A molecule of A2E is formed by enzymatic hydrolysis from its precursor: *N*-retinyl-*N*-retinylidene-phosphatidylethanolamine (A2PE). In addition to A2E, such ATR conjugates as a dimer of ATR (ATR-dimer), a conjugate of ethanolamine with the ATR-dimer (ATR-dimer-E), a conjugate of the ATR-dimer with phos-

phatidylethanolamine[5] (ATR-dimer-PE), and dihydropyridine (DHP) derivatives A2-DHP-PE and A2-DHP-E have been characterized to the present time.[9]

It is believed that A2E makes the main contribution to the general image of human eye fundus autofluorescence.[10] The question about the contributions of other fluorophores to the image of eye fundus autofluorescence remains unanswered. Probably, the content of various fluorophores in LG or their ratio can vary during aging for different forms of retina degeneration and at different stages of the pathological process. For example, it is known that at Stargardt´s disease the fluorescence maximum is shifted to the short-wavelength region.[11] This shift could be explained by the accumulation of oxidized forms of A2E or other ATR conjugates.[12]

Thus, it is evident that the fluorescence properties of all fluorophores in the LG composition and their contribution to the general image of eye fundus autofluorescence should be studied in detail.[1]

The purpose of the present work is to study the spectral properties of individual fluorophores (or their groups) and to estimate their contribution to the total spectrum of LG fluorescence using the methods of fluorimetry, spectrophotometry, and HPLC.

Experimental

Reagents from Sigma, Sigma—Aldrich, Fluka, and Komponent-reaktiv and plastic 1-mL disposable tubes and disposable pipettes (Eppendorf) were used. Solvents of chromatographic purity from Sigma and Fluka were used for HPLC.

Isolation of lipofuscin granules. The granules were isolated from RPE of eyes of 17—70-year donors. After cornea for transplantation was isolated, the eyes of the donors without any ophthalmologic pathologies were received for studies from the Eye Tissue Bank of the Sv. Fyodorov Eye Microsurgery Complex. All stages of isolation were carried out under the muted light.

Lipofuscin granules were isolated and purified by the described methods.[3] The samples contained $\sim 2 \cdot 10^7$ granules per 1 mL of a 0.1 *M* phosphate buffer (pH 7.3).

Preparation of a chloroform extract from lipofuscin granules. A twofold excess of a chloroform—MeOH (2 : 1) mixture was added to a suspension of LG in a 0.1 *M* phosphate buffer with pH 7.2—7.4, and the mixture was stirred with an electrical stirrer for 2 min and then incubated for 10 min at 4 °C. The mixture was centrifuged on an MLW K 26 D centrifuge (680 g, 10 min, 4 °C). The bottom chloroform phase was taken with a syringe and transferred into a flask. The obtained chloroform extract was evaporated with a water-jet pump. For the further chromatographic analysis, MeOH (200 μL) was added to the resulting precipitate.

***N*-Retinyl-*N*-retinylidene ethanolamine (A2E)** was synthesized by the earlier described procedure,[13] and its purity was monitored by HPLC on a Knauer chromatograph (Germany).

Absorption and fluorescence spectra recording. Absorption spectra were recorded on a Shimadzu UV-1700 spectrophotometer (Japan). Fluorescence spectra were recorded on a Shimadzu RF-5301PC fluorimeter (Japan).

High-performance liquid chromatography (HPLC). Derivatives of ATR and other polyenes were separated on a Knauer chromatograph (Germany) with the column Diasfer 120 C18 (4 × 250 mm, sorbent size 5 μm) using linear gradient elution in the system: from 80% MeCN—20% water (+0.05% TFA) to 100% MeCN for 20 min at a flow rate of 1.5 mL min^{-1}. Measurements were carried out at the wavelength 430 nm using a K-2501 spectrophotometric detector (Knauer, Germany). The fluorescence of the detected bands was detected on an RF-10A-xl fluorescence detector (Shimadzu, Japan), which was consecutively connected with the spectrophotometric detector of the chromatograph. The surface areas of the peaks in the chromatogram were calculated and processed using the EuroChrom 5.05 chromatographic program. An inaccuracy for each sample was determined from three separately obtained chromatograms for each individual sample. The percentage content of components in the mixture was calculated as the amount of the total surface area of all peaks, *i.e.*, the relative content of components. The linear distribution was obtained by data processing and, therefore, Student´s coefficient was chosen for further calculations of the reliability.

Results and Discussion

Absorption spectra of a suspension and a chloroform extract of lipofuscin granules. For the component analysis of the fluorescence properties of LG, lipophilic compounds were extracted from them with chloroform. The absorption spectra of a suspension of LG in a phosphate buffer (*1*) and a chloroform extract from LG (*2*) are shown in Fig. 1. It is seen that the chloroform fraction contains substances absorption maxima at 280, 340, and 430 nm. Radiation with these wavelengths was used for the excitation of fluorescence of the studied products. In addition, the wavelength 488 nm was also used for fluorescence excitation, since in ophthalmologic practice it is used in a commercial confocal scanning laser ophthalmoscope (Heidelberg, Germany) for the registration of the general image of eye fundus autofluorescence.

Chromatographic analysis of a chloroform extract from LG (by absorption). Absorption spectra of individual fluorophores or their groups. In order to separate a mixture of

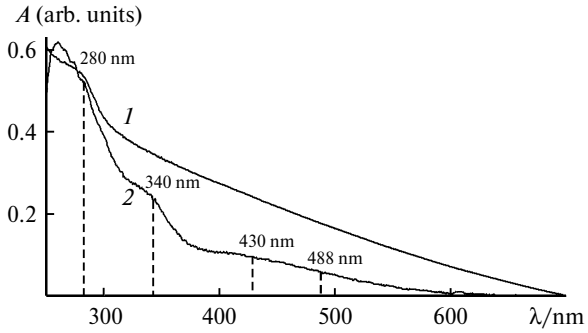


Fig. 1. Absorption spectra of an LG suspension in the phosphate buffer (*1*) and a chloroform extract from LG (*2*).

substances in a chloroform extract from LG into particular components or their groups, we performed the chromatographic analysis of the extract and determined the spectral characteristics of individual components. The chromatogram of a chloroform extract from LG detected at the wavelength 430 nm is presented in Fig. 2. It is seen that the sample contains many components. We identified A2E (peak *15*), *iso*-A2E (peak *17*), and oxidized forms of A2E (peaks *12* and *13*).[14] The nature of peaks *1*–*11* with shorter retention times is unknown. We have earlier[12] observed the appearance of similar peaks in the chromatogram upon the irradiation of synthetic A2E with the white light for 2 h. It can be assumed that these substances are the products of photodegradation or photooxidation of A2E. Fractions *18* and *19* can contain such ATR conjugates as A2-DHP-PE and A2-DHP-E,[9] as well as ATR-dimer, ATR-dimer-E, and ATR-dimer-PE (see Ref. 5).

For the determination of the spectral properties of the detected substances, fractions *a*—*h* were taken during the chromatographic separation of substances in a chloroform extract and their absorption spectra were recorded (Fig. 3). The intensity of the absorption spectra and their maxima in Fig. 3 show that compounds A2E (peak *15*), *iso*-A2E (peak *17*), and oxidized forms of A2E (fraction *f*, peaks *12* and *13*) are predominant products by absorption in the visible spectral region (400—500 nm). Fractions *a*—*e* absorb in the UV region to a greater extent and at 400 nm to a smaller extent. Fraction *h* consists of a group of substances absorbing both in the short-wavelength (at 300 nm) and long-wavelength (at 500—600 nm) spectral regions.

Chromatographic analysis of a chloroform extract from LG (by fluorescence). Fluorescence spectra of individual fluorophores or their groups. For the determination of the fluorescence properties of detected products presented in the chromatogram (see Fig. 2), chromatographic analysis was carried out on an RF-10A-XL fluorescence detector (Shimadzu, Japan) consecutively connected to the spectrophotometric detector of the chromatograph (Fig. 4). To compare the fluorescence properties of different fluorophores, the chromatograms were obtained at different wavelengths of excitation (340, 430, and 480 nm) and emission (470, 500, and 540 nm, respectively). A comparison of the absorption and fluorescence chromatograms (see Figs 2 and 4, respectively) clearly shows that the fluorescence intensity of A2E is not prevailing over other products, as it is accepted presently.[15,16] Moreover, for excitation with the wavelength 480 nm the fluorescence of A2E almost disappears at a detection wavelength of 540 nm. This indicates that more polar than A2E products with the smallest retention times can make the main contribution to the general image of fundus autofluorescence (excitation wavelength 488 nm).

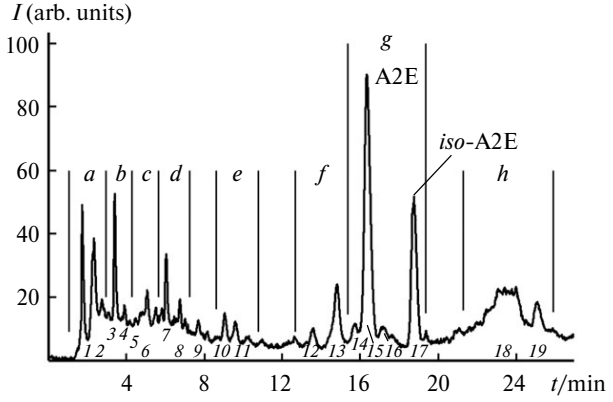


Fig. 2. Chromatogram of a chloroform extract of LG. Detection at a wavelength of 430 nm. Vertical lines restrict the groups of peaks, which were collected as particular fractions *a*—*h* for the determination of the spectral characteristics of fluorophores in the extract.

As can be seen from the chromatogram (see Fig. 4), the highest fluorescence intensity is observed for the products with the characteristic retention times shorter than 3 min (see Fig. 2, peaks *1* and *2*). The contribution of fluorescence of these products remains predominant at different excitation (340, 430, and 480 nm) and emission (470, 500, and 540 nm, respectively) wavelengths (see Fig. 4, Table 1). However, it should be mentioned that the chromatograms by fluorescence do not give rather objective image of the contribution to the total fluorescence of the chloroform extract of individual fluorophores, because they were detected at one wavelength (470, 500, or 540 nm). In this case, fluorescence of some products at

Table 1. Relative distribution of the fluorescence intensity of particular components of the chloroform extract from lipofuscin granules at various wavelengths of excitation (ex) and emission (em)

Peak[a]	Peak surface area (arb. units)[b]		
	340 (ex)/470 (em)	430 (ex)/500 (em)	480 (ex)/540 (em)
1	40.96±12.01	31.08±6.09	17.02±3.02
2	4.42±1.02	6.61±2.31	13.26±2.09
3	4.56±1.14	—	—
4	7.89±1.16	1.01±0.34	0.19±0.05
5	1.81±0.21	0.08±0.003	—
6	2.79±1.01	0.06±0.01	0.15±0.02
7	0.08±0.002	0.02±0.002	—
8	0.68±0.04	—	—
9	0.74±0.02	—	0.07±0.02
10	0.63±0.11	0.09±0.01	0.02±0.003
11	0.14±0.01	—	0.09±0.02
12	0.39±0.04	—	0.08±0.03
13	0.67±0.06	—	—
14 (A2E)	10.12±2.25	2.89±1.07	0.06±0.01
15	3.89±1.34	1.79±0.67	0.96±0.26

[a] Peak number in the chromatogram (Fig. 4).
[b] The statistical mean data were calculated by Student´s criterion. The reliability was $p < 0.05$.

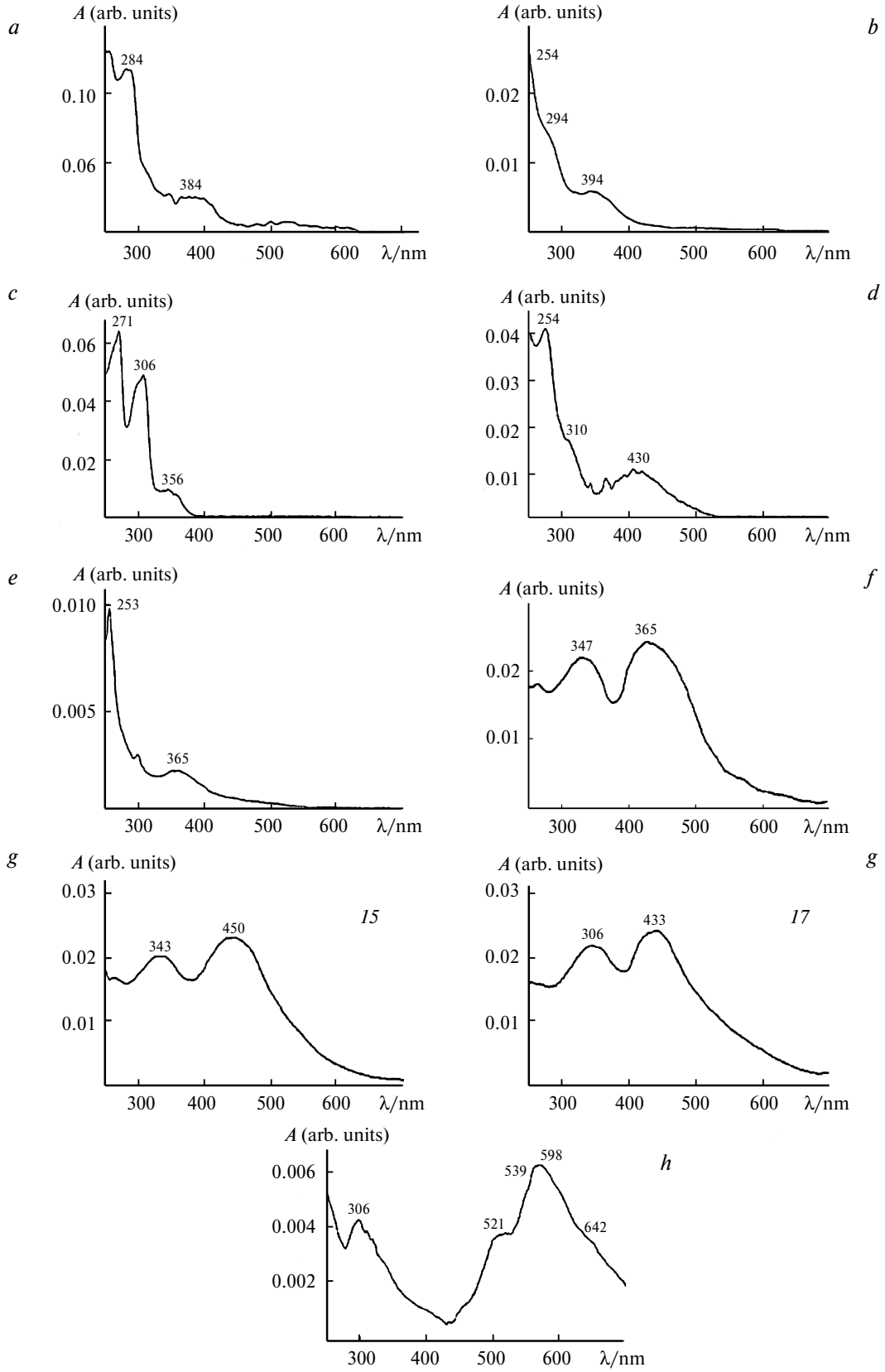


Fig. 3. Absorption spectra of particular fractions *a*—*h* obtained by chromatography of a chloroform extract of LG. Figures 15 and 17 designate the numbers of particular peaks in the chromatogram (see Fig. 2). The spectra were obtained by collecting the fraction from one chromatographic analysis.

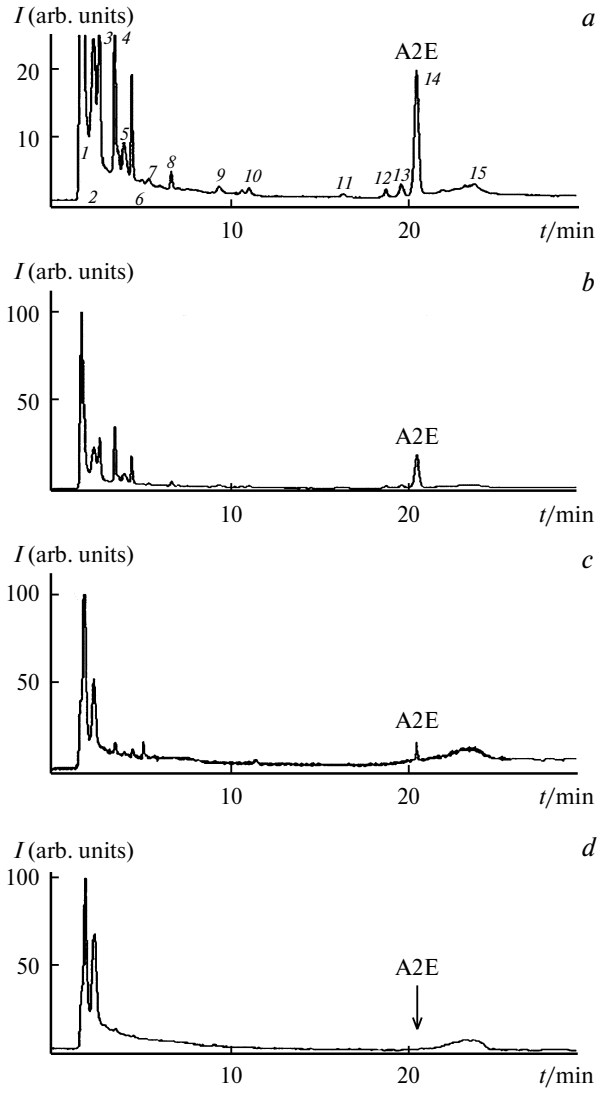


Fig. 4. Fluorescence chromatograms of a chloroform extract of LG: excitation at the wavelength λ = 340 (*a*, *b*), 430 (*c*), and 480 nm (*d*); emission detection at the wavelength 470 (*a*, *b*), 500 (*c*), and 540 nm (*d*).

these parameters of fluorescence excitation and registration is not detected at all.

For more detailed analysis of the spectral characteristics of particular fluorophores and groups of fluorophores in a chloroform extract, the detected products presented in the chromatogram (see Fig. 2) were divided into groups. According to this, eight fractions (*a*—*h*) were taken (see Fig. 2).

The fluorescence properties of particular fractions were studied upon the excitation of their fluorescence with different wavelengths (280, 340, 430, and 488 nm). Although the natural lens almost does not pass the short-wavelength light shorter than 380—400 nm, we also used for fluorescence excitation such wavelengths as 280 and 340 nm for the determination of the spectral properties of all studied fluorophores.

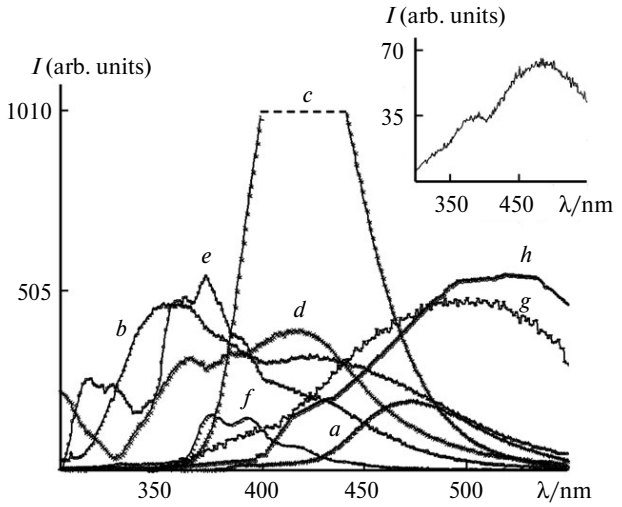


Fig. 5. Fluorescence spectra of fractions of a chloroform extract of LG (excitation wavelength 280 nm); designations of the fractions correspond to those presented in the chromatogram (see Fig. 2). The fluorescence spectrum of a chloroform extract of LG upon excitation at the wavelength 280 nm is presented in inset.

Excitation at the wavelength 280 nm. Figure 5 shows that fraction *c* (maximum at 420 nm) possesses the strongest fluorescence. Fractions *a* and *f* have the lowest fluorescence intensity. All other fractions fluoresce with approximately equal intensity, regardless of the intensity by absorption in this spectral region (see Fig. 3).

Excitation at the wavelength 340 nm. Figure 6 shows that fractions *c* (maximum at 420 nm) and *f* (main maxima at 375 and 392 nm) have the strongest fluorescence. The fluorescence bands of these products are rather nar-

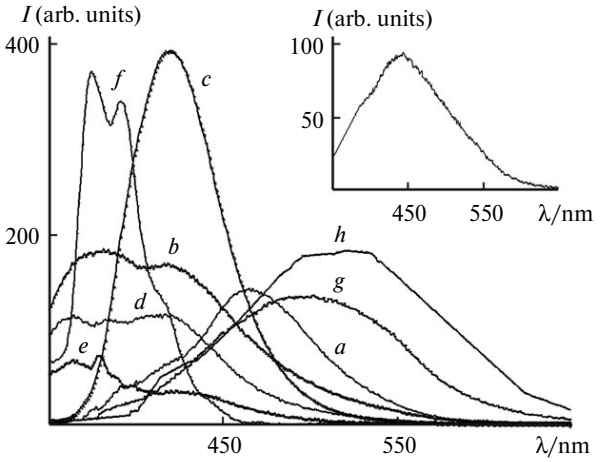


Fig. 6. Fluorescence spectra of fractions of a chloroform extract of LG (excitation wavelength 340 nm); designations of the fractions correspond to those presented in the chromatogram (see Fig. 2). The fluorescence spectrum of a chloroform extract of LG upon excitation at the wavelength 340 nm is presented in inset.

row compared to those of other products. Their spectral ranges are restricted by approximately 450 nm for fraction *f* and 500 nm for fraction *c*. Therefore, these products do not substantially contribute, most likely, to the general image of eye fundus autofluorescence.

Excitation at the wavelength 430 nm. The ratio of fluorescence intensities changes noticeably when particular fractions of a chloroform extract from LG are excited with a wavelength of 430 nm. It is seen from Fig. 7 that fraction *a* exhibits the highest intensity. At the same time, almost no fluorescence is detected for the fluorescence excitation of fractions *c*, *d*, *e*, and *f*. It can be concluded that the contribution of the studied products from these fractions absorbing in the short-wavelength spectral region to the general image of eye fundus autofluorescence is insubstantial. At the same time, according to the literature data,[17,18] the products in fractions *c*, *d*, *e*, and *f* contain oxidized cytotoxic forms of the fluorophores, first of all, the oxidized forms of A2E and *iso*-A2E.

The oxidized forms of A2E identified in our work (see Fig. 2, peaks *12* and *13*) belong to fraction *f*, which does not almost fluoresce upon the excitation with the light of 430 nm and a longer-wavelength light.

Fraction *b* also gives low fluorescence intensity upon the excitation with the wavelength 430 nm.

Of analyzed fractions *a*—*h*, the longest-wavelength fluorescence belongs to A2E, *iso*-A2E (fraction *g*), and the last fraction *h* (maxima at 500 nm). These fractions make almost equivalent contributions to the total fluorescence spectrum of a chloroform extract of LG.

Thus, when considering the spectral region of 500—600 nm, where the general image of eye fundus autofluorescence is detected, one should take into account the contribution of both fluorophores A2E and *iso*-A2E and the products from fractions *a* and *h*. The nature of products in fraction *a* is unknown. As for fraction *h*, it can contain, judging from the literature data, such ATR conjugates as A2-DHP-PE and A2-DHP-E,[9] as well as ATR-dimer, ATR-dimer-E, and ATR-dimer-PE (see Ref. 5).

Excitation at the wavelength 488 nm. The light with the wavelength 488 nm was used to determine the contribution of individual fluorophores to the general image of eye fundus autofluorescence detected at the clinic with a commercial confocal scanning laser ophthalmoscope (HRA-2, Heidelberg, Germany).

Figure 8 shows that in the spectral region of 500—600 nm the fluorescence of fractions *a* and *h* possesses a comparable intensity with fraction *g* (A2E and *iso*-A2E). In addition, the total intensity of all other fractions also can contribute noticeably to the total fluorescence spectrum.

The diagram of fluorescence intensity distribution of various fractions upon the excitation with the wavelength 488 nm and emission detection at the wavelength 570 nm is presented in Fig. 9.

It follows from this diagram that A2E is not the main fluorophore of LG. The products detected by the chromatographic analysis at the initial retention times (shorter than 3 min) and the products eluted after A2E and all its isomeric forms (in 20 min) also contribute substantially to the general image of eye fundus autofluorescence.

The chromatographic analysis of a chloroform extract from LG using the fluorescent detector gave the chromatographic image of fluorescence of separated components with the *unexpected* ratio by fluorescence intensity of particular chromatographic bands (see Fig. 4). For instance, the fluorescence intensity of the bands corresponding to A2E and its isoforms upon excitation with the wave-

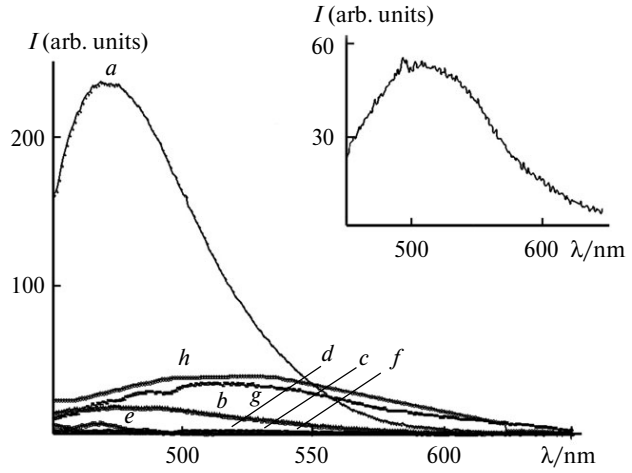


Fig. 7. Fluorescence spectra of fractions of a chloroform extract of LG (excitation wavelength 430 nm); designations of the fractions correspond to those presented in the chromatogram (see Fig. 2). The fluorescence spectrum of a chloroform extract of LG upon excitation at the wavelength 430 nm is presented in inset.

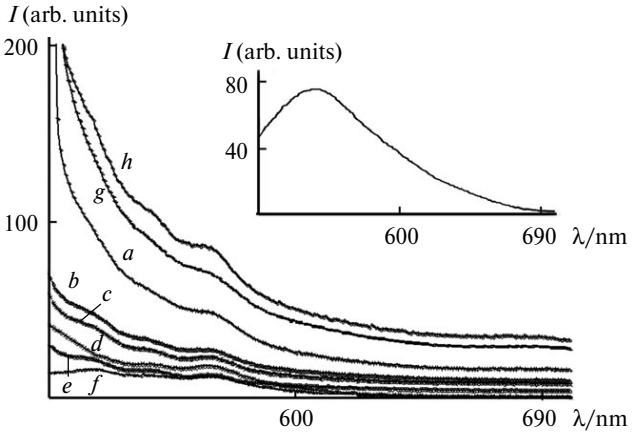


Fig. 8. Fluorescence spectra of fractions of a chloroform extract of LG (excitation wavelength 488 nm); designations of the fractions correspond to those presented in the chromatogram (see Fig. 2). The fluorescence spectrum of a chloroform extract of LG upon excitation at the wavelength 488 nm is presented in inset.

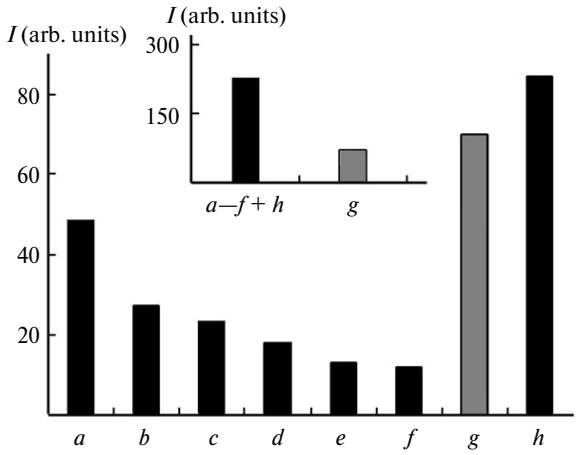


Fig. 9. Diagram of the intensity distribution of fluorescence excited at the wavelength 488 nm for fraction *a—h* (see chromatogram in Fig. 3) in point 570 nm. Inset: the diagram of the intensity distribution of fluorescence excited at the wavelength 488 nm for summated fractions *a—f*, *h* and individual isomers A2E (fraction *g*) (see chromatogram in Fig. 2) in point 570 nm.

lengths 430 and 488 nm turned out to be much lower compared to the bands corresponding to the products with retention times shorter than 3 min, *i.e.*, more polar than A2E. As mentioned above, these products can present, most likely, the products of photooxidation or photodegradation of A2E (see Ref. 12). These results indicate that the detailed spectral analysis of the composition of fluorophores in LG should be performed, since this is A2E which is considered up to presently to be the major fluorophore in LG giving a considerable contribution to the general image of eye fundus autofluorescence on which the new non-invasive diagnostic technique in ophthalmology is based.[10] Moreover, one of the main problems of this modern and promising method is the determination of contributions of different fluorophores to the general image of autofluorescence.[1]

Thus, the component analysis of the fluorophores in a chloroform extract obtained from LG can give valuable information for the improvement of diagnostic potentialities of this method. In this work, we carried out the chromatographic and spectral analyses of individual fluorophores or their groups in a chloroform extract from LG. The results obtained indicate that many components, being ATR conjugates of different nature can contribute to the general image of eye fundus autofluorescence. In this case, fluorophore A2E is not predominant. Upon the excitation of the extract with the wavelength 488 nm, the fluorescence comparable with that of A2E is given by at least two more groups of substances having retention times in chromatographic analysis shorter than 3 min and longer than 20 min, respectively. The nature of the first group of fluorophores (retention times shorter than 3 min) is unknown. It can be assumed that these fluorophores represent the products of photodegradation or photooxidation of A2E (see Ref. 14). As for the second group of fluorophores (retention times longer than 20 min), it can contain, judging from the literature data, such ATR conjugates as A2-DHP-PE and A2-DHP-E,[9] as well as ATR-dimer, ATR-dimer-E, and ATR-dimer-PE.[5]

Thus, the data obtained suggest that A2E is not the predominant fluorophore in a chloroform extract from LG. Probably, it is not predominant in the general image of eye fundus autofluorescence registered upon excitation with the wavelength 488 nm when a commercial confocal scanning laser ophthalmoscope is used at the ophthalmologic clinic.

The task of further studies is the determination of the chemical nature of the first group of fluorophores (retention times shorter than 3 min), which are likely the products of photooxidation and photodegradation of A2E. Urgency of these studies is also dictated by our earlier results showing the tendency for increasing the total relative content of the A2E photooxidation and photodegradation products in LG with aging.[12] It could be reasonable to assume that at some visual pathologies the total relative content of the A2E photooxidation and photodegradation products would be more substantial.

The results of the analysis can be used to enhance the informative content of the new of non-invasive diagnostic technique in ophthalmology: the method of eye fundus autofluorescence.

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