Chapter 5

Absorption of the Eye Lens and Macular Pigment Derived from the Reflectance of Cone Photoreceptors

N.P.A. Zagers and D. van Norren

Submitted

J. van de Kraats and J.J. Vos are acknowledged for critically reading the manuscript.

Abstract:
We measured the amplitude of the directional component of the bleached fundus reflectance, the so called optical Stiles–Crawford effect, as a function of wavelength. The directional reflectance originates from within the outer segments of the photoreceptors. Thus, only two anterior absorbers are of importance: macular pigment and the crystalline lens. Analysis of spectra obtained in pseudophakes established that the cone photoreceptors act as spectrally neutral reflectors. The reflectance spectra, expressed in density units, resembled the macular pigment density spectrum. Studying age effects in the lens of normal subjects resulted in a description of the optical density of the lens in terms of a “young” and an “aged” template. The young template represents the pigment 3-HKG, which dominates the light absorption in young eyes and decreases with age. The aged template represents the pigments accumulating in the lens with age. The total optical density increased with age, but it was
lower in the wavelength region 500–650 nm than was previously assumed on base of psychophysical studies. Analysis of the spectra also provided precise individual estimates of the optical density of macular pigment. Finally, we observed a decrease of the photoreceptor reflectivity with age, possibly reflecting a degradation of the photoreceptors.

5.1 Introduction

Light reflected from the fundus exhibits directional and spectral properties (Chapters 3 and 4).\textsuperscript{1–5, 15–23, 72, 82} The directional property has become known as the optical Stiles–Crawford effect. In a typical experiment dedicated to reveal this phenomenon, a small spot centered at the fovea is illuminated through a small entrance pupil. The distribution of the reflected light, as measured in the dilated pupil, shows a bell shaped directional component superimposed on a flat background.\textsuperscript{19} The maximum reflectance is generally located slightly nasal to the center of the pupil. Van de Kraats et al. argued that the flat background represents diffusely reflected and backscattered light.\textsuperscript{3} This diffuse component originates from various layers in the fundus, with a major contribution from the choroid.\textsuperscript{3} The directional reflectance stems from the highly organized outer segments of the photoreceptors, containing a large number of parallel membranes or disks.\textsuperscript{3} Recently, we developed a new instrument based on an imaging spectrograph, capable of measuring both the spectral and directional aspects of fundus reflectance in the wavelength range 420–790 nm (Chapter 3).\textsuperscript{72} This enables a separation of the spectra for the diffuse and directional component of fundus reflectance.

In the past decades, several models for the spectral reflectance of the fundus have been published.\textsuperscript{1–5} The development of these models was recently reviewed by Berendschot et al.\textsuperscript{45} One of the major challenges has been to provide a proper description of the diffusely scattered non-directional light. The directional reflectance received much less attention, except in the earlier mentioned model by van de Kraats et al.\textsuperscript{3} The present paper concentrates on the amplitude of the directional reflectance
from the bleached fovea, as a function of wavelength. With regard to possible reflectors, we assume that the only source of reflectance resides in the outer segments of the photoreceptors.\textsuperscript{3} Thus, all the other reflecting layers, \textit{e.g.}, the inner limiting membrane, the retinal pigment epithelium, the choroid, and the sclera, play no significant role. With regard to possible absorbers, in a condition with the photopigments bleached, light is only absorbed in the eye media, mainly the crystalline lens, and in the macular pigment, located predominantly along the axons of the nerve cells in front of the receptors.\textsuperscript{29, 30}

To eliminate uncertainty about age effects in the spectral absorption of the crystalline lens, we first studied the spectral behavior of the directional component in pseudophakic subjects. Their implant lenses required only a minor spectral correction. The optical density spectrum of the macular pigment is available from the literature and contains little uncertainty. The pseudophakes’ spectra corroborated an assumption in the model of van de Kraats \textit{et al.},\textsuperscript{3} \textit{viz.} that the reflection from the photoreceptors is spectrally flat. With that answer available, we could achieve the second aim of this study, \textit{viz.} an analysis of age effects in the crystalline lens. For that purpose we obtained data on a group of healthy subjects, aged 18 to 64 years. In addition, analysis of the directional reflectance yielded precise values for the macular pigment density. Finally, we studied receptor reflection as a function of age.

5.2 Methods

5.2.1 Instrument

The instrument has been described in detail elsewhere (Chapter 3).\textsuperscript{72} A number of essentials are summarized below. The key element of the instrument was an imaging spectrograph with its entrance slit conjugate to the pupil plane. Spectral range of the spectrograph was 420–790 nm. The slit of the spectrograph defined a horizontal 0.8 × 12 mm exit pupil, placed over the dilated pupil of the subject. The coil of a 30 W halogen lamp was relayed to the pupil plane of the eye, defining a 0.8 × 1.2 mm entrance pupil. This small entrance pupil was placed centered with, and below the bar shaped exit pupil. Their separation was 0.7 mm. The intensity of the measuring light entering the eye was 1.10 × 10\textsuperscript{6} Troland. A 2.8 deg diameter spot centered at the fovea was illuminated. Ametropia was corrected for with the front lens focus. The subject fixated on a central cross hair. The central 1.9 deg of the illuminated spot was sampled. A chin rest and temple pads connected to a headrest were used to maintain head position. A video channel was available for observation of both retina and pupil.
With the imaging spectrograph, a spectral image of the bar shaped field in the pupil plane was projected on the chip of a cooled CCD camera. For calibration purposes, reference spectral images were obtained from a diffuse reflecting surface painted with Eastman 6080 white paint and a dark black cloth in each session. For each pixel of the CCD, the counts in the measurement and reference spectral images were converted to an equivalent percentage reflectance $R$, and an estimate of the error in reflectance. Prior to the calculation of reflectance, the images were binned and interpolated to 5 nm spectral and 0.1 mm spatial resolution. The array of reflectance values can be looked upon as the distribution of light within a narrow bar placed over the pupil, versus wavelength.

### 5.2.2 Protocol

The research followed the tenets of the Declaration of Helsinki and was approved by the local Medical Ethics Committee. The purpose was explained at the beginning of the experiment, and written informed consent was obtained. The first part of the study included five eyes of five subjects with an intra-ocular lens made of polymethylmethacrylate with bonded UV-absorbers. These pseudophakic subjects were aged 39, 57, 58, 61, and 84 years. The second part of the study included 39 eyes of 39 normal subjects aged 18–64 years; 23 of them were typically young adults, in the age bracket 18–27 years, the other 16 were roughly evenly spread over the 32–64 years age bracket. The pupil of one eye was dilated with one or two drops tropicamide 0.5%. Subjects were instructed to fixate a cross hair centered on the illuminated field at all times. The entrance beam was focused in the pupil plane by moving the headrest back and forth. Fixation and front lens focus were checked in the retinal image. We then searched for the maximum in the directional reflectance ($i.e.$, the maximum of the optical Stiles–Crawford effect) using the measuring field. During the search, continuously directional profiles near 540 nm were read from the CCD. At that wavelength the directionality showed up prominently. In the horizontal direction in the pupil plane (along the spectrograph slit) the maximum position was readily observed in a profile plot on a computer display. The maximum in the vertical direction was found by manual search. While moving the headrest vertically, the actual profile was compared by eye to the highest profile till then. The search procedure took approximately 2 minutes. In this period, visual pigments were bleached away by the measuring light at approximately 97%.$^{25}$ At the optimal entrance position 5 spectra were obtained. In the pseudophakic subjects between 3 to 10 spectra were obtained. The integration time was set at 1.0 s.
5.2.3 Separation of the Directional Reflectance

For each 5 nm band in the range 420–650 nm, the 5 measured profiles were independently fitted with

\[ R(x) = B + A_{\text{dir}} 10^{-\rho (x-x_c)^2}, \]  

(5.1)

with \( R \) the fractional reflectance, \( x \) the location in the pupil plane in mm, \( B \) the non-directional background, \( A_{\text{dir}} \) the amplitude of the directional reflectance, \( \rho \) a measure for the directionality, and \( x_c \) the center position.\(^{18-20}\) Parameters \( B, A_{\text{dir}}, \rho, \) and \( x_c \) were fitted with a least-squares method.\(^{62}\) Each data point was assigned a weight 1 over its error squared. Data included in the fit met two conditions: distance to either of the pupil edges more than 1 mm, and distance from the entrance beam less than 3 mm. Because the background rose steeply above 600 nm, it became increasingly difficult to achieve a reliable fit. For this reason, the wavelength range was limited to 650 nm. The subsequent steps of the analysis concentrated on the amplitude \( A_{\text{dir}} \) versus wavelength. This amplitude, given as a percentage reflectance, was converted to optical density \( D_{\text{dir}} = -10 \log A_{\text{dir}}. \)  

Two sources contributed to the error in \( D_{\text{dir}} \). The first \((\sigma D)\) resulted from the error in \( A_{\text{dir}} \), which was estimated in the fit with Eq. (5.1). The second \((\sigma E)\) was the experimental variation between the five measurements within a series, which was estimated from the standard deviation of \( D_{\text{dir}} \) at 540 nm. The total error \( \sigma_{D-\text{tot}} = (\sigma D^2 + \sigma E^2)^{\frac{1}{2}} \). Data points were rejected when the calculated amplitude was larger than 5% or smaller than zero, or when the error \( \sigma D \) was larger than 0.5. These choices were somewhat arbitrary, but guaranteed that a minority of cases, where the fit with Eq. (5.1) failed, was discarded in the next steps of the analysis. After application of these criteria, the mean density within a series was calculated for each subject, which improved the signal to noise ratio. We also produced mean density data representing a typical young and typical aged eye. Among the youngest and oldest subjects, ten subjects having the best signal to noise ratio at the shorter wavelengths were selected. Five measurements were included for each subject. In the young and aged group the mean ages were 20 and 56 years, the standard deviations were 1.3 and 5 years.

5.3 Experiment I: Pseudophakes

5.3.1 Model for the Directional Reflectance

Following van de Kraats et al.,\(^3\) we assumed that the directional reflectance originates from a location within the outer segments of the cone photoreceptors. Before reaching the receptor layer, and after being reflected, light is absorbed in the macular
pigment and the ocular media. These considerations led to a model for the amplitude of the directional component:

\[ A_{\text{dir}}(\lambda) = R_{\text{recep}}(\lambda) 10^{-2[D_{\text{mac}}\alpha_{\text{mac}}(\lambda) + D_{\text{media}}\alpha_{\text{media}}(\lambda)]} \]  \hspace{1cm} (5.2)

with \( A_{\text{dir}}(\lambda) \) the amplitude of the directional component versus the wavelength \( \lambda \) in nm, \( R_{\text{recep}}(\lambda) \) the reflectance of the photoreceptors, \( D_{\text{mac}} \) the optical density of macular pigment at 460 nm, \( \alpha_{\text{mac}}(\lambda) \) the optical density spectrum of macular pigment normalized to unity at 460 nm, \( D_{\text{media}} \) the optical density of the eye media at 420 nm, and \( \alpha_{\text{media}}(\lambda) \) the optical density spectrum of the eye media normalized to unity at 420 nm. Since the retina was nearly fully bleached by the measuring light, absorption in the visual pigments could be neglected. The macular pigment optical density spectrum has a maximum near 460 nm, so this wavelength was preferred for normalization. The optical density spectrum of the media increases continuously with decreasing wavelength, the normalization wavelength is somewhat arbitrary here.

In van de Kraats et al.’s model, reflectance from the stack of disks was postulated to be a spectrally neutral source of reflectance.\(^3\) We based our model fit on this assumption, that is, we too assumed \( R_{\text{recep}}(\lambda) \) independent of wavelength. The optical density spectrum of macular pigment was obtained from the equation:

\[ \alpha_{\text{mac}}(\lambda) = f_{\text{norm}} \left( 0.32 \exp[-0.0012(436 - \lambda)^2] + 0.32 \exp[-0.0012(480 - \lambda)^2] - 0.123 \exp[-0.0012(458 - \lambda)^2] + 0.12042 \exp[-0.006(457 - \lambda)^2] \right), \]  \hspace{1cm} (5.3)

with \( \lambda \) the wavelength in nm.\(^85\) The above equation was derived by Walraven\(^85\) to represent the macular pigment data in the review by Vos.\(^83\) We incorporated the scalar \( f_{\text{norm}} \), set to 1/0.35, which normalized the equation to unity at 460 nm. In Fig. 5.1A, this macular pigment template is compared with several data sets taken from the literature, all normalized to unit density at 460 nm.\(^25, 65, 83, 84\)

Absorption and scattering losses in the natural eye media, \textit{e.g.}, the cornea,\(^87–89\) the lens capsule,\(^90\) the aqueous humor,\(^87, 88\) and the vitreous humor,\(^87, 88\) were considered negligible in the wavelength region 420–650 nm. The media absorption term in Eq. (5.2) was replaced by a minor correction for absorption in the intraocular lens. A typical optical density spectrum for an intraocular lens was taken from an Alcon product information booklet.\(^86\) A small neutral optical density was dropped; the optical density at 650 nm was set to zero. Fig. 5.1B depicts discrete data points for the
5.3. EXPERIMENT I: PSEUDOPHAKES

Figure 5.1: (A) Optical density spectra of macular pigment, taken from the literature and normalized to unity at 460 nm. Solid triangles, literature review by Vos \(^8^3\) (1972); squares, literature review by Wyszecki and Stiles \(^2^5\) (1982); open triangles, data by Stockman \textit{et al.} \(^8^4\) (1999); circles, data by Brown, cited by Delori \textit{et al.} \(^6^5\) (2001). The solid curve was calculated with the template Eq. (5.3) by Walraven. \(^8^5\) Note that the spectra are not independent, the reviews by Vos and by Wyszecki and Stiles largely rely on the same data. (B) The optical density spectrum of an intraocular lens adapted from an Alcon product information booklet. \(^8^6\) The solid curve produced with Eq. (5.4) closely follows the discrete data points.
intraocular lens density, which could be empirically described (solid curve) by:

\[ D_{\text{IOL}} \alpha_{\text{IOL}}(\lambda) = 0.0838 \exp\left[-\frac{\lambda - 420}{36.6}\right] + 8.55 \exp\left[-\left(\frac{\lambda - 380}{12.9}\right)^2\right]. \]  

(5.4)

After insertion of the intraocular lens absorption term, and by taking minus the logarithm to base 10 on the left and right side of Eq. (5.2), the model becomes a linear combination of three terms:

\[ D_{\text{dir}}(\lambda) = C_r + 2D_{\text{mac}} \alpha_{\text{mac}}(\lambda) + 2D_{\text{IOL}} \alpha_{\text{IOL}}(\lambda), \]  

(5.5)

with \( D_{\text{dir}}(\lambda) \) the optical density equivalent of the amplitude \( A_{\text{dir}}(\lambda) \), \( C_r \) a measure for the spectrally neutral reflection of light by the photoreceptors, and \( D_{\text{mac}}, \alpha_{\text{mac}}(\lambda), \) and \( D_{\text{IOL}} \alpha_{\text{IOL}}(\lambda) \) as defined above. This model, with only two free parameters \( C_r \) and \( D_{\text{mac}}, \) was fitted to the density data with a least-squares method.\(^62\) Each value \( D_{\text{dir}}(\lambda) \) was assigned a weight 1 over its total error.

5.3.2 Results

The upper part of the panels A-E in Fig. 5.2 depicts the amplitude of the directional reflectance converted to a density, versus wavelength for the five pseudophakic subjects. The solid curves depict model fits of Eq. (5.5) to the data. In the upper part of Fig. 5.2F the mean of the five subjects’ data is given. The solid curve is a model fit with Eq. (5.5) to this mean data. All density spectra reflect the shape of the macular pigment density spectrum. Above 540 nm the data suggest no variation with wavelength. The mean single pass optical density of the macular pigment was 0.52. The spectrally neutral reflectance of the cone layer can be read from the average long wavelength value 2.5, which converts to \( R_{\text{recep}} = 10^{-2.5} \), corresponding to 0.32%. At the bottom of each panel in Fig. 5.2 the differences between the data and the model fit are shown. In general, the model provides a good fit to the data and validates the assumption that the reflectance of the receptors is spectrally neutral.

5.4 Experiment II: Normal Subjects

5.4.1 Model with Pokorny et al.’s Lens Templates Applied to Young and Aged Group

When making the transition from the pseudophakes’ model to a model for normal eyes, absorption in the natural crystalline lens is introduced as a new element. We adopted, in a first try, Pokorny et al.’s model for the optical density of the lens as a
5.4. EXPERIMENT II: NORMAL SUBJECTS

Figure 5.2: (A-E) Top: The amplitude of the directional reflectance converted to a density $D_{dir}(\lambda)$, for the pseudophakic subjects. The symbols represent the mean density. Error bars indicate the total errors in the mean. The subjects’ ages and the number of averaged spectra are indicated. The solid curves depict model fits of Eq. (5.5) to the data. Bottom: The differences with the fit at an enhanced scale. (F) The same results for the mean of the five subjects’ data. All the six spectra reflect the optical density spectrum of macular pigment, c.f., Fig. 5.1A.
function of age.\textsuperscript{64} This model describes lens absorption in terms of a template with a fixed density of 0.30 at 420 nm ($\alpha_{\text{lens-\text{na}}}$), and a template with a density affected by aging ($\alpha_{\text{lens-\text{a}}}$). The model becomes a linear combination of four terms:

\[
D_{\text{dir}}(\lambda) = C_r + 2D_{\text{mac}}\alpha_{\text{mac}}(\lambda) + 2D_{\text{lens-\text{a}}}\alpha_{\text{lens-\text{a}}}(\lambda) + 2 \times 0.30 \alpha_{\text{lens-\text{na}}}(\lambda),
\] (5.6)

with $D_{\text{dir}}(\lambda)$, $C_r$, $D_{\text{mac}}$, and $\alpha_{\text{mac}}(\lambda)$ as defined above, $D_{\text{lens-\text{a}}}$ and 0.30 the optical densities of the two lens components at 420 nm, and $\alpha_{\text{lens-\text{a}}}$ and $\alpha_{\text{lens-\text{na}}}$ the lens optical density spectra normalized to unity at 420 nm. The optical density spectra $\alpha_{\text{lens-\text{a}}}$ and $\alpha_{\text{lens-\text{na}}}$ were derived from the templates $T_{L1}(\lambda)$ and $T_{L2}(\lambda)$ as given in Pokorny \textit{et al.}'s Table I, page 1439.\textsuperscript{64} In the model fit implementation, $D_{\text{lens-\text{a}}}$ was rewritten as $10^{\delta_{\text{lens-\text{a}}}}$, which ensured that $D_{\text{lens-\text{a}}}$ remained positive. We applied the model on the mean data for the young and aged eyes (\textit{c.f.}, Methods). These mean data sets had a high signal to noise ratio, which facilitated a thorough test of the model.

### 5.4.2 Comparison of the Model with the Young and Aged Group Data

Figure 5.3A shows the mean density $D_{\text{dir}}$ versus wavelength for the young (bottom spectrum, mean age 20 years) and aged group (top spectrum, mean age 56 years). The symbols represent the mean of in total 50 measurements, 5 for each of the 10 subjects in both groups. The data were not shifted vertically; the optical density in aged eyes is larger at longer wavelengths as well. In contrast to the pseudophakes’ spectra in Fig. 5.2, the normal subjects’ spectra in Fig. 5.3 steeply rise below 450 nm. The imprint of macular pigment absorption on the density spectra is apparent: both

<table>
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<tr>
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<th>Pokorny \textit{et al.}'s model</th>
<th>New templates</th>
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<tr>
<td><strong>Mean, young</strong></td>
<td></td>
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<tr>
<td>$D_{\text{mac}}$</td>
<td>$0.443 \pm 0.015$</td>
<td>$D_{\text{mac}}$</td>
</tr>
<tr>
<td>$R_{\text{recep}}$ (%)</td>
<td>$1.04 \pm 0.02$</td>
<td>$R_{\text{recep}}$ (%)</td>
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<tr>
<td>$D_{\text{lens-\text{na}}}$</td>
<td>$0.30$ (fixed)</td>
<td>$D_{\text{young}}$</td>
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<tr>
<td>$D_{\text{lens-\text{a}}}$</td>
<td>$0.11 \pm 0.02$</td>
<td>$D_{\text{aged}}$</td>
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<tr>
<td><strong>Mean, aged</strong></td>
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<tr>
<td>$D_{\text{mac}}$</td>
<td>$0.54 \pm 0.03$</td>
<td>$D_{\text{mac}}$</td>
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<tr>
<td>$R_{\text{recep}}$ (%)</td>
<td>$0.58 \pm 0.02$</td>
<td>$R_{\text{recep}}$ (%)</td>
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<tr>
<td>$D_{\text{lens-\text{na}}}$</td>
<td>$0.30$ (fixed)</td>
<td>$D_{\text{young}}$</td>
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<tr>
<td>$D_{\text{lens-\text{a}}}$</td>
<td>$0.24 \pm 0.04$</td>
<td>$D_{\text{aged}}$</td>
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Figure 5.3: (A) The mean optical density $D_{\text{dir}}(\lambda)$ for 10 young and 10 aged subjects. The error bars, not discernable in most cases, indicate the total errors in the mean. The data were not shifted vertically; the optical density in aged eyes is larger at longer wavelengths as well. The rise in optical density from 450 to 420 nm is due to absorption in the crystalline lens. The dashed curves depict the best fit with the Pokorny et al.\textsuperscript{64} lens aging model. The solid curves represent the simultaneous fit of Eq. (5.8) to both data sets in order to derive two new templates for the absorption in the lens. (B, C) The differences between the mean aged and young data and the model fit based on the Pokorny et al.\textsuperscript{64} lens aging model (triangles) and the model based on Eq. (5.8) (squares). The new templates improved the fit to the data.
spectra show the absorption edge below 520 nm and a slight local maximum near 460 nm.

The dashed curves in Fig. 5.3A represent the best fits with the model Eq. (5.6). Table 5.1 gives values for the model parameters. The dashed model curve shown in comparison with the top data set in Fig. 5.3A clearly has a slope in the wavelength region 540–650 nm, while the data suggests no variation with wavelength within this range. The triangles in Figs. 5.3B and C illustrate the differences between the mean aged and mean young data and the model fits. Small, but systematic differences between the data and the model based on Pokorny et al.’s lens templates are present, in particular for the mean aged data. The discrepancies warranted a search for an improvement of the model.

5.4.3 Derivation of New Templates

The age-related increase in light absorption by the eye lens has been extensively discussed in the literature. The biochemical processes causing this change are probably complex. However, two main physiological aspects have been identified. In young lenses, absorption is dominated by the pigment O-β-glucoside of 3-hydroxykynurenine, or 3-HKG (some authors use 3-OHKG). The optical density spectrum of 3-HKG presents an absorption band near 365 nm, with a tail up to 450 nm. With age, the amount of 3-HKG in the lens decreases. Nevertheless, there is a net increase in lens absorption, because other light absorbing pigments accumulate in the lens. These pigments, probably proteins, have a maximum absorption at 320 nm and show a shallow tail extending considerably further in the visible wavelength range than 3-HKG. As a result of the concomitant decrease in 3-HKG and the accumulation of the other pigments, the absorption maximum shifts from 365 to 320 nm, and a shallow tail shows up in the visible wavelength region.

In view of the physiological considerations described above, and in line with Pokorny et al.’s model, we aimed at optimizing the shape of two spectral templates. The contribution of both templates was allowed to vary with age. For the first template we propose to use the optical density spectrum of 3-HKG. We coin this the “young” template. Figure 5.4A depicts several spectra taken from the literature, normalized to unity at 370 nm. The solid curve in Fig. 5.4A represents a model fit with a single Gaussian in the wavelength range 350–450 nm to the recent 3-HKG spectrum by Heckathorn et al. The model fit represents their data well, but also comes close to Pokorny et al.’s choice for the non-aging component. The latter was multiplied by a scalar to fit the 3-HKG data at 400 nm. The Gaussian curve, after
Figure 5.4: (A) The optical density of 3-HKG, normalized to unity at 370 nm; crosses, Dillon et al.\textsuperscript{91} (1984); squares, Dillon et al.\textsuperscript{92} (1990); circles, Heckathorn et al.\textsuperscript{93} (2001). The diamonds represent the spectrum of 3-hydroxy-kynurenine (3-HK) by Stutchbury et al.\textsuperscript{94} (1993), which is similar to the 3-HKG spectra. Dillon et al.’s (1990) spectrum\textsuperscript{92} was first reported representing 3-HK, but in 1991 Dillon presented the same data as a 3-HKG spectrum.\textsuperscript{95} The spectrum of the eye of a young monkey (triangles) by Gaillard et al.\textsuperscript{40} (2000) is similar to the 3-HK and 3-HKG spectra. The solid curve depicts a single Gaussian fitted to Heckathorn et al.’s 3-HKG data.\textsuperscript{93} The solid triangles represent Pokorny et al.’s non aging template.\textsuperscript{64} It has the same shape as the 3-HKG spectra. (B) Various suggested optical density spectra for the pigments accumulating with age, normalized to unity at 420 nm; squares, Dillon et al.\textsuperscript{92} (1990); crosses, Dillon et al.\textsuperscript{39} (1999), triangles, Gaillard et al.\textsuperscript{40} (2000). The circles depict the Pokorny et al.\textsuperscript{64} template affected by aging. The curves drawn through the above data sets represent a fit with the tail of a single, or the sum of two Gaussians centered at 320 nm. The thick solid curve depicts the newly proposed aged template Eq. (5.9).
normalization to unity at 420 nm, was taken as the young template:

$$\alpha_{\text{young}}(\lambda) = 6.09 \exp \left[ - \left( \frac{\lambda - 370}{37.2} \right)^2 \right]. \quad (5.7)$$

As to the second lens absorption template the literature is divided. Three spectra of the pigments accumulating with age, as well as Pokorny et al.’s template for the component affected by aging are depicted in Fig. 5.4B.\(^{39,40,64,92}\) Making a choice for a particular curve was difficult. First, there were large differences between the three sources. Second, all three were obtained from excised material. Third, the spectra were obtained with a light source containing UV and blue light, but were not corrected for fluorescence in the lens. Finally, the spectra were corrected for scattered light by fitting the data in the wavelength range 600–700 nm with a power law. This correction was then extrapolated to shorter wavelengths and subtracted from the data. An error in this correction is expected when it is extrapolated down to 400 nm.

Because a spectrum for the pigments accumulating with age was not readily available, we derived a new template, which we will refer to as the “aged” template. First, we sought a mathematical function suitable for representation of the aged template in the model fit. There is consensus in the literature that the lens spectrum gradually rises with decreasing wavelength, asymptotically approaches a constant value at longer wavelengths, and has little or no fine structure. A priori, the tail of a Gaussian seemed appropriate for describing the lens spectra. To assure that this choice does not pose too large a limitation on the outcome of the aged template, we first tested our assumptions on the literature data in Fig. 5.4B. It proved possible to fit the three spectra as well as Pokorny et al.’s template for the component affected by aging with a single, or with the sum of two Gaussians. The resulting model curves are drawn through the data in Fig. 5.4B. Since the pigments accumulating with age have an absorption maximum at 320 nm (not shown here), we fixed the center position of the Gaussians to this value.\(^{40}\) Dillon et al.’s data\(^{92}\) (1990) do not show the tail at longer wavelengths; they could be fitted well with a single Gaussian.

In the second step, we used the same mathematical model to derive the aged template from the mean aged and young group data. With the young template known, and with a single Gaussian at 320 nm, Eq. (5.6) transforms to:

$$D_{\text{dir}}(\lambda) = C_r + 2D_{\text{mac}} \alpha_{\text{mac}}(\lambda) + 2D_{\text{young}} \alpha_{\text{young}}(\lambda) + 2D_{\text{aged}} \exp \left[ \left( \frac{320 - 420}{\Delta} \right)^2 \right] \exp \left[ - \left( \frac{\lambda - 320}{\Delta} \right)^2 \right], \quad (5.8)$$

with \(D_{\text{dir}}(\lambda), C_r, D_{\text{mac}},\) and \(\alpha_{\text{mac}}(\lambda)\) as defined earlier, \(D_{\text{young}}\) and \(D_{\text{aged}}\) the optical density of the young and aged component at 420 nm, \(\alpha_{\text{young}}(\lambda)\) the young template
given by Eq. (5.7), and the expression following \( D_{\text{aged}} \) a single Gaussian with a free width \( \Delta \), representing the aged template. The first exponential serves to normalize the aged template to unity at 420 nm. In the model fit implementation, \( D_{\text{young}} \) and \( D_{\text{aged}} \) were rewritten as \( 10^{\delta_{\text{young}}} \) and \( 10^{\delta_{\text{aged}}} \), which ensured that \( D_{\text{young}} \) and \( D_{\text{aged}} \) remained positive. The model, with free parameters \( C_r, D_{\text{mac}}, \delta_{\text{young}}, \delta_{\text{aged}}, \) and \( \Delta \) was simultaneously fitted to the mean young and aged data with a least-squares method. Parameters \( C_r, D_{\text{mac}}, \delta_{\text{young}}, \) and \( \delta_{\text{aged}} \) were optimized for each group separately. The width of the aged template \( \Delta \) was optimized as well, but kept equal for the young and aged group.

The solid curves in Fig. 5.3A represent the model fit with Eq. (5.8). The model fit parameters are given in Table 5.1. We also evaluated a model fit with the sum of two Gaussians at 320 nm, but found no improvement. The aged template is characterized by the parameter \( \Delta \), which was 76.0 ± 2.4 nm. Normalized to unity at 420 nm, it is given by:

\[
\alpha_{\text{aged}}(\lambda) = 5.65 \exp \left[ -\left( \frac{\lambda - 320}{76.0} \right)^2 \right].
\]

(5.9)

It is shown in Fig. 5.4B as a thick solid curve. It runs an even steeper course than Dillon et al.’s (1990) data. The differences between the mean \( D_{\text{dir}}(\lambda) \) data and the model, plotted as squares in Figs. 5.3B and C, have diminished to the order of 0.02 density units in almost the full wavelength range.

5.4.4 Model with New Templates Applied to Individual Data

The final model is arrived at after insertion of the two new templates in Eq. (5.6):

\[
D_{\text{dir}}(\lambda) = C_r + 2D_{\text{mac}}\alpha_{\text{mac}}(\lambda) + 2D_{\text{young}}\alpha_{\text{young}}(\lambda) + 2D_{\text{aged}}\alpha_{\text{aged}}(\lambda),
\]

(5.10)

with \( D_{\text{dir}}(\lambda), C_r, D_{\text{mac}}, \) and \( \alpha_{\text{mac}}(\lambda) \) as defined before, the templates \( \alpha_{\text{young}}(\lambda) \) and \( \alpha_{\text{aged}}(\lambda) \) given by Eqs. (5.7) and (5.9), and \( D_{\text{young}} \) and \( D_{\text{aged}} \) the optical densities of the two lens components at 420 nm. The model Eq. (5.10) was fitted to the data for individuals with a least-squares method. The lens densities \( D_{\text{young}} \) and \( D_{\text{aged}} \) were rewritten as \( 10^{\delta_{\text{young}}} \) and \( 10^{\delta_{\text{aged}}} \). Free parameters were \( C_r, D_{\text{mac}}, \delta_{\text{young}}, \) and \( \delta_{\text{aged}} \).

The upper parts of the panels in Fig. 5.5 depict the amplitude of the directional reflectance, converted to a density, versus wavelength for a sample of six normal subjects spanning the full age range. The solid curves depict model fits of Eq. (5.10) to the data. At the bottom of each panel in Fig. 5.5 the differences between the data and the model fit are shown at an enhanced scale. Generally a good fit of the model to the data was obtained. Figure 5.6 shows the density of the young component, the aged component, and the total lens density versus age at 420 nm. The solid curves
Figure 5.5: (A-F) Top: The amplitude of the directional fundus reflectance, converted to a density, versus wavelength for a sample of six normal subjects spanning the full age range. The symbols represent the mean density within a series of five measurements. Error bars indicate the total errors in the mean. Ages are indicated above each spectrum. The solid curves depict model fits of Eq. (5.10) to the data. Compared with Fig. 5.2, the spectra steeply rise below 450 nm. The additional optical density is caused by absorption in the crystalline lens. Bottom: The differences between the data and the model fit.
Figure 5.6: (A) The density of the young and (B) the aged components, and (C) the total lens density versus age at 420 nm. The error bars indicate the statistical error estimated in the model fits. The solid curves depict linear regression lines, their parameters are given in Table 5.2. The density of the young component decreased with age. The density of the aged component strongly increased with age. The total optical density of the lens slightly increased with age.
The density of the young component $D_{\text{young}}$ (Fig. 5.6A), representing 3-HKG, decreased with age. In some of the oldest subjects $D_{\text{young}}$ reached zero. The density of the aged component $D_{\text{aged}}$ (Fig. 5.6B), representing the pigments accumulating with age, strongly increased with age. In some of the young subjects, it was absent. As a result of the two concomitant changes, the total lens density at 420 nm (Fig. 5.6C)
5.5. DISCUSSION

Table 5.2: Parameters derived from linear regression with age.

<table>
<thead>
<tr>
<th></th>
<th>Intercept</th>
<th>Slope (10^{-3} year^{-1})</th>
<th>R</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_{\text{young}}$</td>
<td>0.42 ± 0.04</td>
<td>−6.4 ± 1.2</td>
<td>−0.66</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>$D_{\text{aged}}$</td>
<td>−0.08 ± 0.04</td>
<td>9.4 ± 1.1</td>
<td>0.81</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>$D_{\text{young}} + D_{\text{aged}}$</td>
<td>0.34 ± 0.03</td>
<td>3.0 ± 0.8</td>
<td>0.53</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>$D_{\text{mac}}$</td>
<td>0.44 ± 0.05</td>
<td>2.3 ± 1.3</td>
<td>0.28</td>
<td>0.09</td>
</tr>
<tr>
<td>$R_{\text{recep}}$ (%)</td>
<td>1.12 ± 0.07</td>
<td>−11 ± 2</td>
<td>−0.70</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

slightly increased with age. The total optical density of the eye media as a function of age can be described with a combination of the regression models given in Table 5.2, and the two known templates:

$$D_{\text{media}}(\lambda, A) = (0.42 - 0.0064 A) \alpha_{\text{young}}(\lambda) + (-0.08 + 0.0094 A) \alpha_{\text{aged}}(\lambda),$$ (5.11)

with $D_{\text{media}}(\lambda, A)$ the total optical density, $A$ the age in years, and $\alpha_{\text{young}}(\lambda)$ and $\alpha_{\text{aged}}(\lambda)$ given by Eqs. (5.7) and (5.9).

The model fit also provided individual estimates of the optical density of the macular pigment and the reflectivity of the photoreceptors. Figures 5.7A and B depict both parameters versus age. The circles represent the data for the thirty-nine normal subjects. The crosses represent the data obtained in the five pseudophakic subjects in Section 5.3. The solid curves represent linear regressions to the normal subjects’ data. Parameters for the regression analysis are also given in Table 5.2. Macular pigment (Fig. 5.7A) showed no significant change with age ($P = 0.09$). The mean macular pigment optical density was 0.51. The between subjects standard deviation was 0.13. In the normal subjects, the reflectance of the photoreceptors (Fig. 5.7B) strongly decreased with age. The reflectance ranged from 1.19% to 0.38%. The reflectance of the photoreceptors in the pseudophakes did not show any sign to deviate from the downward trend.

5.5 Discussion

We measured spectra for the amplitude of the directional component of fundus reflectance. This type of spectral reflection could be described with a simple model. The single reflecting layer resides in the outer segments of the photoreceptors, and light is only absorbed in the crystalline lens and in the macular pigment. Model analysis of the pseudophakes’ spectra validated the assumption that the source of directional reflectance is spectrally neutral. The second part of the study concentrated on
the age changes in the absorption of the lens. We first tested Pokorny et al.’s model for absorption in the crystalline lens. We found small but significant differences between their model and our mean data for young and aged observers. Therefore, we derived a new lens aging model. Similar to Pokorny et al.’s model, it comprises a combination of two templates: a “young” template representing 3-HKG, and an “aged” template representing the pigments accumulating in the lens with age. A new element is that the young component decreased with age; the old component increased with age. Both changes are reported in the literature. In addition, we observed a hitherto unreported decrease of the photoreceptor reflectivity with age.

5.5.1 Directional Reflection From the Layer of Cone Photoreceptors

An important assumption throughout this paper was that the source of the directional reflectance resides in or near the photoreceptor layer, as was proposed before by van de Kraats et al. Gorrand and Delori suggested that the retinal pigment epithelium also plays a role. The tips of the outer segments of the photoreceptors are intertwined with the microvilli of the retinal pigment epithelium cells. This alternative assumption would lead to practically the same model for the directional reflectance. Burns et al. measured the distribution of the fluorescence in lipofuscin in the pupil plane and found no directionality. This indicates that recapture of light from behind the receptors does not play a major role. The pseudophakes’ spectra (Fig. 5.2) closely resembled the shape of the macular pigment optical density spectrum. This indicates that the source of directional reflectance lies behind the nerve fiber layer. Because no other retinal absorbers, e.g., no melanin or blood, were required to explain the data, reflectance from the choroid can be excluded. This pinpoints the source of directional reflectance to the anterior layers of the retinal pigment epithelium, the receptor layer, or a combination of both.

None of the spectra (Figs. 5.2, 5.3, and 5.5) varied strongly with wavelength above 540 nm. There are no pigments in front of the receptors absorbing in this wavelength range. Below 540 nm absorption by the macular pigment and the eye media come into play. But, because the model satisfactorily described the data, the assumption that the directional reflectance is spectrally neutral below 540 nm as well seems tenable. This is in line with a model calculation of the electrodynamics of a receptor cell by Piket-May et al. They demonstrated that the waveguide properties of the bleached photoreceptors are independent of wavelength. Experimentally this is corroborated by the fact that the spectral sensitivity of the photoreceptors does not deviate from the spectral absorption of their visual pigments. In a previous paper we analyzed the directionality, a measure for the peakedness of the distribution in the
It was found that the waveguide directionality of the cone photoreceptors, to first order, is independent of wavelength, in line with Piket-May et al.’s results. Recently, assessment of the waveguide properties of single photoreceptor cells became possible in vivo. It would be worthwhile to examine the amplitude of the reflectance and its directionality of individual cells as a function of wavelength.

5.5.2 Lens Aging, Objective, in vivo

Objective, in vivo assessment of light absorption in the lens requires a source of reflectance in the eye with known properties. Johnson et al. and Savage et al., following a method originally devised by Said and Weale, measured the amount of light reflected at the back of the lens (the fourth Purkinje image). Van Norren and Tiemeijer, Delori and Burns, van de Kraats et al., and Zagers et al. (Chapter 3) relied on a model analysis of light reflected from the retina. Xu et al. followed a similar approach using the spectral reflectance of the optic disk. Thus far the spectral reflectance models could only be made more or less compatible with experimental data by assuming, without really understanding, diffusely scattered light. Our analysis, with its split up in a directional and non-directional component, removes this difficulty. Assessment of the directional component as a function of wavelength provides a unique method for studying light absorption in the living eye lens. Unfortunately, the lens is an inhomogeneous structure and the central and marginal path lengths are different. In the present experiments the entrance beam was confined to a small region near the center of the pupil, but the exit beam typically spanned 5–6 mm of the pupil. As a result, the lens optical density represents an average over the pupil with most weight placed on the region centered with the maximum of the optical Stiles–Crawford effect. Several authors have proposed corrections for the differences in path length of central and marginal rays. It is not straightforward to implement these corrections, because the maximum of the optical Stiles–Crawford effect and the center of the lens in general do not coincide. The former has a unique location in different individuals. Furthermore, the shape of the lens is not uniquely determined and a purely geometrical correction would not do justice to the inhomogeneous distribution of the absorbing pigments in the lens. Therefore, we have neglected this second order correction.

Analysis of the spectra of the normal subjects provided individual estimates for the optical density of the pigment 3-HKG and the pigments accumulating with age. Many authors have suggested that these or two similar components are the main factors determining lens absorption, yet a quantitative analysis with use
Figure 5.8: Comparison of lens optical density spectra for (A) observers aged approximately 20 years and (B) observers aged approximately 50 years. The solid curves represent the present model calculated with Eq. (5.11). Data obtained with the Purkinje image method: open triangles, Johnson et al. (1993), age groups 21–30 and 41–50 years; solid triangles, Savage et al. (2001), for observers with an average age of 24 and 50 years. Psychophysical data: circles, Pokorny et al.’s (1987) model; dashed curves, Savage et al.’s (1993) model; solid squares, Savage et al. (2001), in the same observers with an average age of 24 and 50 years that provided the objective data represented by the solid triangles.

of the known 3-HKG absorption spectrum was, to our knowledge, never published. The decrease of the young component (Fig. 5.6A), the aged component (Fig. 5.6B), and the increase of the total lens density at 420 nm with age (Fig. 5.6C) have been reported.40,64,92,96,97,99–101 The absence of 3-HKG in some of the oldest lenses is also in agreement with earlier findings.101 The aged component also reached zero, but not
5.5. DISCUSSION

Surprisingly only in some of the youngest subjects. In a previously published study on the spectral reflectance of the fovea (Chapter 3), we applied Porkorny et al.’s model for assessment of lens density, and found zero density for the component affected by aging in some of the youngest subjects. In addition, van den Berg and Felius fitted the optical density of excised lenses with the Porkorny et al. model and found zero density for the component affected by aging in some of the youngest specimens. In contrast, the Porkorny et al. model predicts a considerable contribution of the component affected by aging, even in the age range 20–30 years.

The total lens density at 420 nm (Fig. 5.6C) only slightly increased with age. The two underlying processes compete and the increase of the aged component just overcompensates the decrease of the young component. The increase of the total optical density at 420 nm with age was obscured by the large variation between subjects of comparable age. This large spread within age groups was encountered in previous studies on large groups of subjects as well. As in our data, it was regularly observed that the highest densities at age 20 are as high as the lowest at age 60. Gaillard et al. suggested that the spectral changes in the lens are the result of chemical or photochemical modifications, not biological aging. The environment, in particular the light history, poses different levels of chemical or photochemical stress to an individual. Thus, age might be a poor determinant for the changes in the lens.

In Figs. 5.8A and B the solid curves, predicted with Eq. (5.11) for subjects aged 20 and 50 years, are compared with data from the literature. Johnson et al.’s data is depicted by the open triangles. Savage et al. (2001) provided data for a group of subjects with mean age 24 and 50 years (solid triangles). We conclude that there is good agreement between our data and the results derived with the objective Purkinje image method. Both data sets show no changes with age above 540 nm.

5.5.3 Lens Aging, Psychophysics

The curves in Fig. 5.8 for the total lens density can also be compared with psychophysical data. The circles in Figs. 5.8A and B were calculated with Pokorny et al.’s lens aging model for a 20 and 50 year old observer. The data were shifted vertically to normalize the optical density to zero at 550 nm. The dashed curves were calculated with Savage et al.’s (1993) psychophysical model for observers aged 20 and 50 years. The solid squares represent psychophysical data obtained by Savage et al. (2001) in the same observers of average age 24 and 50 years that provided the objective data (solid triangles). The psychophysical data exhibit systematically
higher optical densities for wavelengths below 550 nm than the objective data. Savage et al. (2001) discussed this difference and suggested that an additional absorbing pigment is located in front of the peripheral receptors. More extensive research with both types of measurement in the same set of subjects would be helpful to clarify this interesting phenomenon.

Pokorny et al.'s model predicts age-related changes at wavelengths as high as 640 nm. As stated before, our data suggests no variation with age above 540 nm. For the discussion of the differences between the present results and the Pokorny et al. model, it is of interest to review the fundamentals of the latter. One of the starting points was the compromise curve in the first edition of the book “Color Science” by Wyszecki and Stiles (1967). For wavelengths above 420 nm, this curve was mainly based on donor eyes obtained by Ludvigh and McCarthy (n = 4) and Weale (n = 2; ages 48 and 53 years). Ludvigh and McCarthy measured four complete eyes with an average age of 62 years. They also measured the lenses of these eyes separately, as well as 5 lenses with an average age of 21.5 years. From this data, they calculated the transmission of a whole eye, as if it had contained a young lens. One of the lenses used by Weale had been invaded by a melanoma, and in both lenses a control experiment clearly demonstrated postmortem effects. Two decades later, van Norren and Vos reviewed the literature available at that time and compared it with the Wyszecki and Stiles compromise curve. They concluded that this curve was adequate for the visual region, but too low for the ultraviolet. Also, they derived a new ocular density curve from the CIE scotopic sensitivity function and the absorption curve of human rhodopsin. Below 430 nm, the new curve followed the literature data more closely. Above 430 nm, the Wyszecki and Stiles compromise curve was maintained. On their turn, in the second edition of “Color Science”, Wyszecki and Stiles (1982) referred to van Norren and Vos and concluded that an update of the compromise curve from the first edition was not required. Pokorny et al. took Wyszecki and Stiles’ (1982) curve as the starting point for their lens aging model. Analysis of the color matching data for subjects aged 16–55 years published by Stiles and Burch yielded the spectral shape of the partition of lens optical density affected by aging $L_1$ and a fixed residual component $L_2$. The fixed component reached zero at 460 nm. From our analysis of the cited literature sources, it can be concluded that Pokorny et al.’s total optical density $T_L$ in the visible wavelength range relates to only six donor eyes. As a consequence, for wavelengths above 460 nm, the shape of the aging component also relates to the same six donor eyes. In our view, this provides a rather weak experimental basis for the model in the visible wavelength region in particular at the longest wavelengths. This might at least partly explain why we have found a considerably higher transmission in the wavelength region 500–650 nm.
5.5. **DISCUSSION**

5.5.4 **Macular Pigment**

The model analysis of the normal subjects’ spectra also yielded values for the optical density of the macular pigment. The mean macular pigment optical density in the normal subjects was 0.51, well within the range of values reported in earlier studies (Chapter 3).\(^2,3,72\) Previously, macular pigment was found to be constant with age,\(^121–124\) or to show a minor decline with age.\(^125,126\) In agreement with these findings, no significant trend with age was found. It must be noted that the macular pigment optical densities represented an average by area over the sampled field, in our case 1.9 deg. The macular pigment distribution is highly concentrated toward the center of the fovea. This is a complicating factor when comparing different methods for assessment of macular pigment. Delori *et al.* discussed other difficulties involved when comparing different techniques.\(^65\) As was stated before, isolation of the directional component removed many of the difficulties normally encountered in assessment of macular pigment by means of fundus reflectometry.

5.5.5 **Reflectance of the Photoreceptors**

The reflectivity of the receptors (Fig. 5.7B) strongly decreased with age. From the model Eq. (5.2) it can be inferred that this cannot be distinguished from an increase in spectrally neutral light losses in the eye media. The observed loss in reflectivity between age 20 and 60 years corresponds to the accumulation of a single path optical density of 0.14 density units. Psychophysical studies found forward-scattered light to be independent from wavelength.\(^127,128\) When scattered outside the measurement field this scattered light is lost for detection. However, for our 1.9 deg field, the scatter losses seem too small to explain the entire neutral component. Van den Berg and IJspeert measured on average only 0.09 density units absorption at 700 nm in donor lenses, with the major part of them having cataract.\(^129\) In a similar study on normal lenses aged 59 ± 20 years, van den Berg and Felius found 0.04 ± 0.04 density units at 700 nm.\(^114\) In view of this low value, an increase of a spectrally neutral absorbing pigment is considered an unlikely explanation of the density increase.

The number of cones in the fovea is stable with age,\(^130\) but the disks in the outer segments undergo a gradual but major disruption.\(^131\) This phenomenon is the most probable cause of the loss of reflectivity with age. To our knowledge, the present data represent the first observation of this kind *in vivo.*