The Color Difference in Orbital Fat

Bryan S. Sires, MD, PhD; John C. Saari, PhD; Gregory G. Garwin, BS; John S. Hurst, PhD, MSc; Frederik J. G. M. van Kuijk, MD, PhD

**Objective:** To identify and quantify carotenoids found in white and yellow orbital fat.

**Methods:** Specimens of nasal (white) and preaponeurotic (yellow) orbital fat were obtained from patients during upper eyelid blepharoplasty. Carotenoids and retinoids were extracted and subjected to spectral and high-performance liquid chromatography analyses.

**Results:** The chromophore content of extracts from unsaponified fat, as measured by absorbance at 425 nm per gram of fat, was 2- to 4-fold higher in preaponeurotic fat than in nasal fat. High-performance liquid chromatography analysis from enzymatically digested fat revealed large amounts of lutein, β-carotene, and retinol and small amounts of other unidentified carotenoids. The amount of β-carotene and lutein in preaponeurotic fat was approximately 4-fold higher than in nasal fat.

**Conclusions:** The higher carotenoid content of preaponeurotic fat might cause it to be more yellow than other orbital fat, and lutein and β-carotene might be selectively absorbed from plasma by preaponeurotic fat.

**Clinical Relevance:** The results provide baseline information for studies of the physiological features of orbital fat in normal and diseased conditions.


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PREAPONEUROTIC orbital fat is more yellow than the white fat from the rest of the orbit (Figure 1). Differences in protein and fatty acid content do not account for this difference in color. Carotenoids are known to be present in the fat of various body regions. These C40 tetraterpenoids absorb blue and UV light, and their localization in the retina of primates gives the macula lutea its characteristic yellow color. To date, their presence in orbital fat has not been demonstrated.

This study compared the carotenoid content of nasal and preaponeurotic fat to determine whether a quantitative difference in carotenoid content could account for the color difference in the regional fat deposits of the superior orbit.

**SPECTRAL ANALYSIS OF EXTRACTS OF FAT PADS**

Hexane extracts of preaponeurotic fat pads were yellow, indicating the lipophilic nature of the chromophore. In preliminary experiments, hexane extracts were purified with a normal-phase HPLC column, which resulted in elution of greater than 80% of the 450-nm absorbing material in a peak at the solvent front. The complex spectrum obtained with this mixture of components could be approximated by the additive spectra of 2 carotenoids (results not shown), providing the first indication that 1 or more carotenoids were responsible for the yellow color. The chromophore content of fat pad extracts, as measured directly in hexane extracts by absorbance at 425 nm per gram of fat pad, was 2- to 4-fold higher in preaponeurotic fat pads than in nasal fat pads (mean ± SD, 2.94 ± 0.78) (Table 1). Quantification of spectra at several other wavelengths yielded similar findings.

**CHROMOPHORE CHARACTERIZATION**

Carotenoids extracted from preaponeurotic and nasal fat pads were analyzed using reversed-phase HPLC after enzymatic digestion of triacylglycerols and extraction into hexane. The results display a variety of components coeluting...
MATERIALS AND METHODS

ORBITAL FAT SAMPLES

Preaponeurotic and nasal fat specimens were obtained during routine upper eyelid blepharoplasty after detailed consent was obtained from each patient. Specimens from 5 patients were used for quantification of the total amount of carotenoid in each of the 2 fat regions. Specimens from another 7 patients were used for high-performance liquid chromatography (HPLC) analysis of the carotenoids found in each fat region. Immediately after retrieval of the specimens, they were placed in a –80°C freezer until analysis.

CAROTENOID QUANTIFICATION

Extraction of Carotenoids

All procedures involving carotenoids were performed under red illumination to minimize photodecomposition of the compounds.

For spectral and normal-phase HPLC analyses, carotenoids were extracted without saponification using hexane. Frozen orbital fat pads were weighed and homogenized at 0°C in 1 mL of MOPS (3-N-morpholinopropane sulfonic acid), 0.1 mol/L, pH 6.5; sodium chloride, 0.1 mol/L; EDTA, 1 mmol/L; and 1 mL of ethanol using a glass-glass tissue homogenizer. Homogenates were extracted 3 times using 4 mL of hexane, and the volume of combined hexane phases was reduced to 1 mL using flowing argon for spectral analysis or to dryness for HPLC analysis.

For reversed-phase HPLC analysis, carotenoids were extracted from fat samples using hexane after enzymatic digestion of the triacylglycerols of the sample. A mixture of digestive enzymes was prepared that contained triglyceride hydrolase, 160 IU/mL (Sigma-Aldrich Co, St Louis, Mo), and cholesterol esterase, 1 IU/mL (Sigma-Aldrich Co), in sodium phosphate buffer, 10 mmol/L, pH 7.4; potassium chloride, 150 mmol/L; diethylenetriaminepentaacetic acid, 2 mmol/L; and 0.25% Triton X-100. Orbital fat specimens (10-20 mg) were homogenized in 1 mL of this mixture, and the homogenate was digested at room temperature overnight. After incubation, 100 µL of 5% sodium dodecyl sulfate was added, and the samples were vortexed for 30 seconds. One milliliter of ethanol containing butylated hydroxytoluene, 50 mg/mL, was added, and the samples were extracted using 3 mL of hexane that contained internal standards.

Continued on next page

Table 1. Comparison of 425-nm Absorbance per Gram of Nasal vs Preaponeurotic Fat

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Nasal Fat (A)</th>
<th>Preaponeurotic Fat (B)</th>
<th>Ratio (B/A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.6</td>
<td>2.6</td>
<td>4.23</td>
</tr>
<tr>
<td>5</td>
<td>1.3</td>
<td>4.1</td>
<td>3.12</td>
</tr>
<tr>
<td>6</td>
<td>1.4</td>
<td>2.9</td>
<td>2.07</td>
</tr>
<tr>
<td>7</td>
<td>2.7</td>
<td>5.8</td>
<td>2.18</td>
</tr>
<tr>
<td>8</td>
<td>2.1</td>
<td>6.6</td>
<td>3.08</td>
</tr>
</tbody>
</table>

*Mean ± SD ratio, 2.94 ± 0.78.

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Preaponeurotic fat of the upper orbit is an important surgical landmark for the surgeon because it is decidedly more yellow than the remainder of the white orbital fat (Figure 1). Nutritional studies have associated yellow fat with carotenoid accumulation, and carotenoids have been noted in fat from several regions of the body. Thus, it is not surprising that our results revealed yellow preaponeurotic fat of the orbit to be relatively rich in carotenoids and retinol. In extracts, the total chromophore content of preaponeurotic fat was as much as 4 times greater than that of nasal fat. The yellow chromophore seemed to be quantitatively extracted into hexane because the extracted residue was colorless. High-performance liquid chromatography analysis yielded similar findings and indicated that the principal chromophores were retinol, lutein, and ß-carotene. We found approximately the same 4-fold excess of total chromophores in unfractionated hexane extracts and in the lutein + ß-carotene + retinol con-
Hexane extracts were dried using anhydrous sodium sulfate before analysis. β-Apo-10'-carotenal methyl oxime was added as an internal standard.

**Reversed-Phase HPLC**

Samples were analyzed using a 4.6 × 40.0-mm C18 column (5-µm particle size) (VYDAC 218TP54; The Separations Group, Hesperia, Calif) and an HPLC fitted with a Beckman 168 diode array detector (Beckman Coulter Inc, Fullerton, Calif). Solvent A consisted of acetonitrile and methanol (85:15 vol/vol), with 0.01% wt/vol ammonium acetate, and solvent B was pure hexane. Elution started with a 97:3 vol/vol mixture of solvents A and B, respectively, at 0.55 mL/min. At 10 minutes, the flow rate was increased linearly to 1.2 mL/min in 5 minutes. At 23 minutes, the flow rate was decreased linearly in 1 minute to 0.55 mL/h. The column was equilibrated at this flow rate for an additional minute before injection of the next sample.

**Normal-Phase HPLC**

Hexane extracts were analyzed using a Supelcosil LC-Si narrow-bore analytical column (2.1 × 150.0-mm, 3-µm particle size) using an instrument (model 1050; Hewlett-Packard Company, San Fernando, Calif) with a solvent-conditioning module, quaternary pump, and diode array detector. Carotenoids were eluted by washing the column using the following solvents (flow rate, 0.5 mL/min): 0 to 10 minutes, 100:0 vol/vol (hexane-acetone); 10 to 30 minutes, linear gradient to 60:40 vol/vol; and 30 to 40 minutes, 100:0 vol/vol. Spectra of selected components were obtained during their elution using the “spectra on the fly” feature of Chemstation A.04.02 software. Spectra of eluted components were compared with published spectra of carotenoids as follows: zeaxanthin, all-trans-lycopene, β-carotene, and β-carotene and lutein.

**Spectral Analysis**

Hexane extracts were analyzed using a UV-visible spectrophotometer (model 640B; Beckman Instruments, Fullerton, Calif). The combined chromophore content of the extract was estimated from the maximum absorbance at 425 nm of the complex spectrum that resulted.

**Table 2. Concentration of Lutein and β-Carotene per Unit of Orbital Fat**

<table>
<thead>
<tr>
<th>Sample No. and Fat Type</th>
<th>Lutein, ng</th>
<th>B/A</th>
<th>β-Carotene, ng</th>
<th>B/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>10A</td>
<td>0.11</td>
<td>2.2</td>
<td>0.15</td>
<td>3.4</td>
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<tr>
<td>10B</td>
<td>0.24</td>
<td>1.9</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>11A</td>
<td>0.56</td>
<td>1.9</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>11B</td>
<td>1.06</td>
<td>1.9</td>
<td>1.65</td>
<td></td>
</tr>
<tr>
<td>20A</td>
<td>1.00</td>
<td>1.9</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>20B</td>
<td>0.22</td>
<td>0.36</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>21A</td>
<td>0.16</td>
<td>4.4</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>21B</td>
<td>0.30</td>
<td>1.9</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>22A</td>
<td>0.05</td>
<td>1.9</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>22B</td>
<td>0.16</td>
<td>3.2</td>
<td>0.13</td>
<td>1.4</td>
</tr>
<tr>
<td>23A</td>
<td>0.11</td>
<td>2.5</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>23B</td>
<td>0.28</td>
<td>4.4</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>24A</td>
<td>0.05</td>
<td>2.5</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>24B</td>
<td>0.22</td>
<td>4.4</td>
<td>0.45</td>
<td></td>
</tr>
</tbody>
</table>

*A indicates nasal fat; B, preaponeurotic fat; and ND, not detectable.

The presence within the orbit of both yellow and white fat is surprising and raises several interesting physiological and functional questions. How can these 2 adjacent fat deposits differ in carotenoid composition? We considered whether anatomical differences between the 2 types of fat might account for the higher carotenoid content. White orbital fat is described as finely lobulated and rich in fibrous tissue, whereas yellow fat is described as fluid, homogeneous, and sparsely vascularized. In addition, preaponeurotic fat is believed to have a different embryonic origin, appearing within the orbit during the second month of gestation, whereas white orbital fat differentiates from embryonic mesenchyme at about the fourth month. Thus, the 2 types of fat differ developmentally and histologically, but the differences offer little insight into the mechanisms of specific carotenoid uptake.
The macula lutea of primate retina concentrates certain plasma carotenoids (lutein and zeaxanthin) and restricts others (lycopene and β-carotene).\textsuperscript{7,8} In addition, metabolism is likely to occur within the macular region, converting lutein to mesozeaxanthin.\textsuperscript{8} The mechanisms responsible for the selective uptake in this tissue are not understood.

Does the accumulation of carotenoids in preaponeurotic fat serve a biological function? Some carotenoids are thought to act as antioxidants by virtue of their ability to quench reactive oxygen species and terminate radical-mediated chain reactions.\textsuperscript{14,15} In addition, within the macula lutea, lutein and zeaxanthin absorb blue light, which might contribute to decreased activation of sensitizing molecules and reduced chromatic aberration in this region.\textsuperscript{15,16} However, neither of these processes seem to be required in the orbital region adjacent to the aponeurosis and muscle of the superior levator muscle.

In summary, this study identifies the yellow chromophores of preaponeurotic orbital fat as carotenoids and retinol and quantifies the differences in chromophore content between this fat and nasal orbital fat. Further studies are needed to understand the mechanism by which this process occurs and whether the increased carotenoid and retinol content in preaponeurotic fat serves a biological function.

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Corresponding author: Bryan S. Sires, MD, PhD, Department of Ophthalmology, University of Washington, Campus Box 356485, Seattle, WA 98195-6485 (e-mail: bsires@u.washington.edu).

REFERENCES