ing the excitation of chlorophyll via the Soret band, using a blue laser (e.g., 407 nm). Similar excitation in combination with the use of specific band-pass filters (420–440 nm and 460–490 nm) could be used to differentiate between chlorophyll *a* and chlorophyll *b*, respectively. Such conditions are suitable for isolating mutants of algal species containing both chlorophyll *a* and *b* or for the examination of protoplasts of higher plants. Finally, mutants of organisms in which carotenoids serve as the major light-harvesting pigments could also be isolated; for example, the level of fucoxanthine, the predominant carotenoid in certain algae, is reflected in a broad fluorescence band centered around 530 nm.

In summary, the four genes isolated thus far and further characterization of their products (11–15) provided some clues to the mechanism of degradation of the phycobilisome, the highly abundant macromolecular structure comprising the cyanobacterial light-harvesting complex. The isolation of novel nonbleaching mutants, however, seems to be a rate-limiting step in the study of the degradation process and its regulation. FACS-mediated selection of nonbleaching mutants will undoubtedly lead to a better understanding of the molecular mechanisms underlying the modulation of light harvesting in response to environmental cues by facilitating the isolation of novel mutants.

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Rapid analysis of oxysterols by HPLC and UV spectroscopy

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Oxidized derivatives of cholesterol are an important group of molecules with potent pharmacological properties (1) that have been implicated in atherosclerosis and other age-related diseases (2). The accurate identification and quantification of oxysterols is complicated by numerous factors, including low sensitivity of detection, interference by other compounds, tedious analytical methods, and instability during alkaline hydrolysis.

Cholesterol and most oxysterols have ultraviolet (UV) absorption maximums below 200 nm and are extremely hydrophobic. This creates a unique chromatography problem because most solvents capable of solubilizing oxysterols (with dielectric constants below 30) absorb light strongly between 190 and 200 nm and their use reduces the sensitivity of detection by UV absorption. Water and acetonitrile have low UV absorption profiles above

190 nm but are poor solvents for oxysterols with dielectric constants of 80 and 36, respectively. A recently published high-performance liquid chromatography (HPLC) technique (3) can separate most oxysterols, but it is complex to perform and uses solvents with strong UV absorption, which reduces the sensitivity of detection.

This protocol resolved the poor solubility problems by using high concentrations of acetonitrile at high temperatures. However, this alone did not resolve the chromatography problems because most commercially available C-18 columns still retained cholesterol and oxysterols, requiring stronger solvents with high UV absorption below 200 nm (methanol) or long elution times that broadened the peaks and reduced the sensitivity of detection. This problem was solved by using the Xterra® RP18 column (Waters, Milford, MA, USA), which is not only more stable at high temperatures than the silica-based C-18 column but also binds

the oxysterols less tightly. This column allows the elution of cholesterol and its oxidized derivatives using acetonitrile in less than 15 min, reducing the peak broadening and providing clean spectral data to aid in their identification.

The sterol analyses were performed using an HPLC system (Model 2790 Separation Module; Waters) controlled with Empower™ Pro Software (Waters). Sterols were detected using a photodiode array detector (Model 996; Waters) equipped with a 4-μL cell. The oxysterols were separated in an Xterra RP-18 column (4.6 × 250 mm) running a gradient starting at 15% 1 mM phosphoric acid in water, 85% to 100% acetonitrile at 60°C for 15 min, and flowing at 1 mL/min. The column was flushed with 100% methanol for 2 min and re-equilibrated with 15:85 (v/v) 1 mM H₃PO₄ water to acetonitrile for 5 min between injections. The spectra were collected between 190–300 nm.

Cholesterol and authentic oxysterol standards (Steraloids, Newport, RI,

USA) previously reported to be present in oxidized low-density lipoprotein (ox-LDL) (4) were separated as described above (Figure 1). Quantification of all of the oxysterols was performed by peak area integration at 200 nm, with the exception of 7-ketocholesterol (7kCh), which is done at 237 nm. The 7kCh is well separated from 7β-hydroxycholesterol (7βHCh), but the sensitivity at 237 nm is almost 4 times greater than at 200 nm. The separation of 7α-hydroxycholesterol (7αHCh) and 7βHCh is not complete but sufficient enough to allow a reasonably accurate quantification either by peak area or by peak height.

To determine how well this protocol would perform with an actual biological sample, 100 μg of human low-density lipoprotein (LDL) were oxidized using 20 μM CuSO₄ as previously described (5). The LDL was purified from human plasma (Calbiochem, San Diego, CA, USA) and contains approximately 36% cholesterol by weight. The oxLDL was hydrolyzed by the addition of 60% KOH

Figure 1. Three-dimensional spectrum of oxysterols commonly found in oxidized low-density lipoprotein. (A) Three-dimensional (3-D) chromatogram showing the separation and spectrum of 25-hydroxycholesterol (25HCh; 2.5 nmol), 7α-hydroxycholesterol (7αHCh; 5 nmol), 7β-hydroxycholesterol (7βHCh; 5 nmol), 7-ketocholesterol (7kCh; 5 nmol), and cholesterol (Ch; 5 nmol). (B) Spectrum contour (enlargement of inset in panel A). (C) Chromatogram at 200-nm wavelength. This is the wavelength used for quantification of all of the sterols except 7kCh, which has a λ_{max} (wavelength at which there is maximum absorbance) at 237 nm. AU, arbitrary units.

Table 1. Results from the Analysis of Oxidized Low-Density Lipoprotein

oxLDL (25 µg)		
Oxysterol	Amount (nmol)	Comments
25HCh	0.13	N.A.
7αHCh	1.20	N.A.
7βHCh	1.23	N.A.
7kCh	1.92	N.A.
Ch	4.02	N.A.
7k-ox-der	~1.74	Based on 7kCh ^a
Others	~1.05	Based on Ch ^b

oxLDL (10 µg)		
Oxysterol	Amount (nmol)	Comments
25HCh	0.06	N.A.
7αHCh	0.42	N.A.
7βHCh	0.42	N.A.
7kCh	0.58	N.A.
Ch	1.5	N.A.
7k-ox-der	~0.7	Based on 7kCh ^a
Others	~0.41	Based on Ch ^b

oxLDL (2 µg)		
Oxysterol	Amount (nmol)	Comments
25HCh	0.009	N.A.
7αHCh	0.082	N.A.
7βHCh	0.089	N.A.
7kCh	0.150	N.A.
Ch	0.29	N.A.
7k-ox-der	~0.124	Based on 7kCh ^a
Others	~0.057	Based on Ch ^b

The quantification of each oxysterol was performed by area integration using the absorption coefficient for genuine standards. The results from the analysis of 10 and 2 µg indicate that the limit of the sensitivity under these conditions is approximately 10 pmol. The percent recovery based on β-sitosterol (internal standard) was 75%, 83%, and 92% for 25, 10, and 2 µg, respectively. oxLDL, oxidized low-density lipoprotein; 25HCh, 25-hydroxycholesterol; 7αHCh, 7α-hydroxycholesterol; 7βHCh, 7β-hydroxycholesterol; 7kCh, 7-ketocholesterol; Ch, cholesterol; 7k-ox-der, unknown oxidized 7kCh derivative; N.A., not applicable.

^aThe quantification of the unknown oxidized 7kCh derivative (7k-ox-der) was performed using the area integration at 277 nm and the absorption coefficient of 7kCh at 237 nm.

^bThe areas for all other unidentified peaks with ultraviolet (UV) profiles similar to cholesterol were added, and the absorption coefficient for cholesterol was used for their quantification. Thus, the quantification of these compounds is inaccurate, and the values are estimates.

(w/v) in methanol at 37°C for 2 h in argon gas. The sample was neutralized by the addition of 2 volumes of 50% acetic acid, and the sterols were extracted with a 50:50 mixture of petroleum ether and dichloromethane. The solvents were evaporated in a stream of argon, and the sterols were dissolved in 100% ethanol. The analysis of 1/4 of the hydrolyzed sample, equivalent to 25 µg of oxLDL, is shown in Figure 2. The plant sterol β-sitosterol was used as an internal standard because it can be easily separated from cholesterol

and its oxidized derivatives.

As previously reported (4), the oxidation of LDL was dominated by the 7-carbon derivatives (Figure 2). The four main sterols found in the oxLDL were 7αHCh, 7βHCh, 7kCh, and cholesterol. Traces of numerous other components including 25-hydroxycholesterol (25HCh) were also detected. Most of the minor peaks seen at 200 nm that have UV spectra similar to cholesterol are likely oxidized derivatives. A main peak with a

Figure 2. Analysis of human oxidized low-density lipoprotein. The extracted sterols from 100 µg [total low-density lipoprotein (LDL)] of hydrolyzed oxidized low-density lipoprotein (oxLDL) were separated as described in the text. (A) A three-dimensional (3-D) chromatogram of the equivalent of 25 µg of oxLDL. The main components of the oxLDL are the 7-carbon-oxidized derivatives, but other components were also detected but not identified. A major peak with a λ_{max} (wavelength at which there is maximum absorbance) of 277 nm is a derivative of 7-ketocholesterol (7kCh) generated during the alkaline hydrolysis. (B) Chromatogram at 200 nm. 25HCh, 25-hydroxycholesterol; 7αHCh, 7α-hydroxycholesterol; 7βHCh, 7β-hydroxycholesterol; Ch, cholesterol; 7kCh-ox-der, unknown oxidized 7kCh derivative; B-Sito, β-sitosterol; AU, arbitrary units.

Figure 3. Analysis of 10 and 2 µg of human oxidized low-density lipoprotein. (A) Chromatogram monitored at 200 nm separating the oxysterols from 10 µg of oxidized low-density lipoprotein (oxLDL). (B) Chromatogram at 200 nm separating the oxysterols from 2 µg of oxLDL. The amounts of each oxysterol are shown in Table 1. 25HCh, 25-hydroxycholesterol; 7αHCh, 7α-hydroxycholesterol; 7βHCh, 7β-hydroxycholesterol; Ch, cholesterol; B-Sito, β-sitosterol; AU, arbitrary units.

retention time of 9.844 min and a $\lambda_{\max}$ (wavelength at which there is maximum absorbance) of 277 nm (Figure 2) was determined to be a derivative of 7kCh, caused by the alkaline hydrolysis. This compound was observed when pure 7kCh was subjected to the alkaline hydrolysis procedure but not when the other oxysterols were similarly treated (data not shown). The loss of 7kCh during alkaline hydrolysis has been previously reported (4), but no published information on the formation and identification of this 7kCh derivative has been found. The identification of this compound by mass spectroscopy is in progress.

The amounts of the oxysterols measured in Figure 2 are shown in Table 1.

The recovery was determined by the measurement of the internal standard β-sitosterol. The amounts of other oxysterols with spectra similar to cholesterol were quantified using the absorption coefficient of cholesterol.

To determine the maximum sensitivity of the technique, the equivalent of 10 and 2 µg of hydrolyzed oxLDL were analyzed. Figure 3 shows the results of the analyses.

The detection limit of the technique (Figure 3) is 10–20 pmol for most oxysterols. The sensitivity may be slightly increased by integrating peaks collected at wavelengths below 200 nm, although this also increases the baseline noise. In summary, this HPLC method provides a fast, simple, and very sensitive technique

to separate, identify, and quantify most of the oxysterols associated with oxLDL.

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