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Reversed phase HPLC of Met^{58} oxidized rhIL-11: oxidation enhanced by plastic tubes

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Abstract

The hydrophobicity of human recombinant interleukin 11 (rhIL-11) with an oxidized Met^{58} residue is nearly identical to the hydrophobicity of native rhIL-11. Consequently, separation of these species using standard gradient elution or isocratic elution is very difficult. Using an optimized, shallow gradient RP-HPLC method, Met^{58} oxidized rhIL-11 could be separated sufficiently from native rhIL-11. The identity of the oxidized form detected with this method was confirmed by peptide mapping with trypsin and endoproteinase Asp-N, N-terminal sequencing and mass spectrometric analysis. This method was employed to determine the effect of disposable laboratory plastic tubes for the oxidation. The amounts of Met^{58} oxidized rhIL-11 were increased when rhIL-11 samples were stored in plastic tubes at 37°C in the dark. Samples stored in polypropylene tubes were oxidized much more than samples stored in polystyrene tubes. Additionally, the oxidation was greatly enhanced when samples were stored in polypropylene tubes exposed to light before rhIL-11 sample storage. The extent of the oxidation was also affected by the sources of polypropylene tubes. A maximum increase in Met^{58} oxidized rhIL-11 was more than 30% when samples were stored at 37°C for 14 days in polypropylene tubes exposed to a daylight fluorescent lamp for 25 days. Consequently, these results indicate that attention should be paid for selection of suitable plastic tubes used for storage of protein samples, and for protection of the plastic tubes themselves from extended exposure to light while in storage.

Keywords: rhIL-11; Methionine; Oxidation; Sulfoxide; Plastic tubes; Polypropylene

1. Introduction

Oxidation of methionine (Met) residue to sulfoxide is a major degradation pathway of protein pharmaceuticals [1]. Met oxidized forms have been detected in many recombinant proteins such as granulocyte colony stimulating factor [2], growth hormone [3], interleukin 2 [4], interferon α-2b [5], relaxin [6,7], and insulin-like growth factor [8].

Interleukin 11 (IL-11) is a multifunctional cytokine which affects the proliferation, differentia-

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tion, and maturation of various types of hematopoietic cells [9,10]. Recombinant human IL-11 (rhIL-11), developed as a pharmaceutical protein for the prevention of thrombocytopenia, is a *des*-Pro IL-11, containing two methionine residues, Met^{58} and Met^{122} [11,12]. The primary structure of rhIL-11 is illustrated in Fig. 1. Since Met^{58} is located on the surface of the molecule, it is much more susceptible to oxidation than Met^{122}, which is buried within the hydrophobic core of the protein [11,12]. Amino acid substitutions by site-directed mutagenesis, and specific alkylation of Met^{58} residue greatly reduce rhIL-11 biological activity, since Met^{58} is involved in the receptor-binding site [12]. Indeed, Met^{58} oxidized rhIL-11 exhibits less than 10% of the in vitro biological activity of native rhIL-11 (Genetics Institute, unpublished data). Therefore, a reliable method to quantitate Met^{58} oxidized rhIL-11 is necessary for quality control of pharmaceutical rhIL-11 products.

Met^{58} oxidized rhIL-11 and native rhIL-11 have nearly identical hydrophobicity. Consequently, they cannot be separated well using standard gradient elution or isocratic elution on RP-HPLC. Therefore, an optimized RP-HPLC method with sufficient resolution to measure Met^{58} oxidized rhIL-11 was developed, and used to assess the effect of plastic tubes for Met^{58} oxidation of rhIL-11.

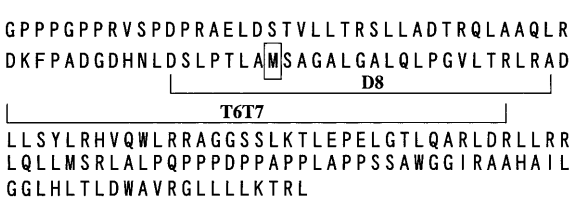

Fig. 1. Primary structure of rhIL-11. The Met^{58} residue is denoted by square. The Met^{58} containing T6T7 peptide resulting from trypsin digestion, and D8 peptide from endoproteinase Asp-N digestion are underlined.

2. Material and methods

2.1. Materials and reagents

Recombinant human IL-11 was obtained from Genetics Institute (Cambridge, MA, USA). L-1-tosylamino-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin was purchased from Worthington Biochemical Corp. (Lakewood, NJ, USA); hydrogen peroxide (H_2O_2) was from Santoku (Tokyo, Japan); catalase was from Wako (Osaka, Japan); trifluoroacetic acid (TFA) was from Pierce (Rockford, IL, USA); polystyrene and polypropylene tubes (17×120 mm) were from Becton Dickinson Co. (Lincoln Park, NJ, USA), Coaster Corp. (Cambridge, MA, USA), and Iwaki Glass (Tokyo, Japan). All other reagents were of analytical grade.

2.2. HPLC analysis

Met^{58} oxidized rhIL-11 analysis was performed by reversed phase HPLC using HP1090M system (Hewlett–Packard). One-hundred μl of 80 μg ml^{-1} rhIL-11 sample was injected on a Waters μBondasphere C4 column (5 μm, 300 Å, 3.9×150 mm). The mobile phases consisted of solvent A (0.1% TFA in 50/50 (v/v) acetonitrile/water) and solvent B (0.1% TFA in 70/30 (v/v) acetonitrile/water). The column was equilibrated initially at 15% B at a flow rate of 1 ml min^{-1}. The separation was performed by a shallow linear gradient of 15–30% B over 30 min at a flow rate of 1 ml min^{-1} at 37°C. Detection was by UV absorbance at 214 nm.

2.3. Characterization of oxidized rhIL-11

Equal volumes of 100 μM rhIL-11 and 100 mM H_2O_2, both in 10 mM sodium phosphate, 300 mM glycine, pH 7.0, were mixed and incubated for 2 h at room temperature. The reaction was stopped by adding 1/100 volumes of 1 mg ml^{-1} catalase solution. The oxidized sample was separated with the RP-HPLC method described above, and the oxidized rhIL-11 peak was fractionated. The fraction was dried with a SpeedVac vacuum concentrator and digested with trypsin at a 1:10 enzyme

to substrate weight ratio in Tris–HCl buffer (pH 8.2) for 20 h at 37°C. Then the reaction was stopped by adding 5 μl of TFA. The trypsin-digested sample was loaded onto a Vydac C18 column (4.6 × 150 mm; Hesperia, CA, USA). The mobile phases consisted of solvent C (0.1% (v/v) TFA in water) and solvent D (0.1% TFA (v/v) in 95% acetonitrile). The column was initially equilibrated at 5% D at a flow rate of 1 ml min^{-1}. The separation was performed by isocratic elution of 5% D for 5 min, followed by a linear gradient of 5–45% D over 27 min and by isocratic elution of 45% D for 2 min at a flow rate of 1 ml min^{-1} at 25°C. Detection was by UV absorbance at 214 nm. Endoproteinase Asp-N mapping was performed according to the method reported by Czupryn et al. [12]. The oxidized peptide was characterized by mass spectrometric analysis on an LCT LC-MS (Micromass, Weesp, The Netherlands) and by N-terminal sequencing by automated Edman degradation conducted on a Procise protein sequencer (PE Biosystems, Foster City, CA, USA).

2.4. Storage of rhIL-11 in plastic tubes

Polypropylene and polystyrene tubes were stored at room temperature with continuously exposed to 1000 lux from either a cool white fluorescent lamp or an artificial daylight fluorescent lamp producing an output similar to the D65 emission standard, for 25 days. As dark controls, the tubes were stored for the same period in positions close to the light-exposed tubes, but wrapped in aluminum foil. After exposure of the tubes to light, 1 ml of 80 μg ml^{-1} rhIL-11 in 10 mM sodium phosphate, 300 mM glycine, pH 7.0 was stored in the tubes at 4 or 37°C for 3, 7 or 14 days in the dark.

3. Results and discussion

3.1. HPLC analysis and characterization of Met^{58} oxidized rhIL-11

rhIL-11 Sample was analyzed using an optimized, shallow gradient, RP-HPLC method (Fig. 2A). The rate of change in the concentration of acetonitrile was approximately 0.1% min^{-1}. Fig. 2A shows a minor peak initially thought to be Met^{58} oxidized rhIL-11. It elutes at a slightly lower acetonitrile concentration than native rhIL-11, and can be clearly separated from the major native rhIL-11 peak under these conditions. To confirm whether the peak was the Met oxidized component, rhIL-11 was oxidized with H_2O_2. RP-HPLC analysis revealed the same peak, but much larger, in samples treated with H_2O_2 (Fig. 2B). This result indicates the peak represents the Met oxidized product of rhIL-11.

To confirm its identity, the peak was collected and analyzed by ESI-MS. The mass of the peak (19 063.6) was 16 amu higher than that of native rhIL-11 (19 047.2). This increase in mass is consistent with oxidation of Met to Met sulfoxide. The collected peak was also digested with trypsin. As a control, unoxidized rhIL-11 sample was also treated with trypsin. The RP-HPLC chromatograms after trypsin digestion are shown in Fig. 3. In the chromatogram of the oxidized sample (Fig. 3B), the T6T7 peak containing the unoxidized Met^{58} residue, which was detected in the chromatogram of the unoxidized sample (Fig. 3A), disappeared, and replaced by a new peak with slightly faster mobility. The N-terminal sequence of the peptide was the same as that of T6T7 peptide and the mass of the peptide (3576.8) was 16 amu higher than that of T6T7 peptide (3560.8). This increase in mass is also consistent with oxidation of Met to Met sulfoxide. These results indicate that the new peak was Met^{58} sulfoxide T6T7 peptide.

In addition to Met residue, His, Cys, Trp and Tyr residues are also potential sites for oxidation [1]. Although Cys, Trp and Tyr are not present, one His residue (His^{48}) exists in the T6T7 peptide. To exclude the possibility that the new peak is due to His^{48} oxidation, Asp-N peptide mapping was performed. As shown in Fig. 4, the two chromatograms of the oxidized and the unoxidized samples were identical except for D8 peptide containing Met^{58} residue, not His^{48}. The mass of the oxidized D8 peak (2707.6) was also 16 amu higher than that of the unoxidized D8 peak (2691.5). These results indicate that the component that

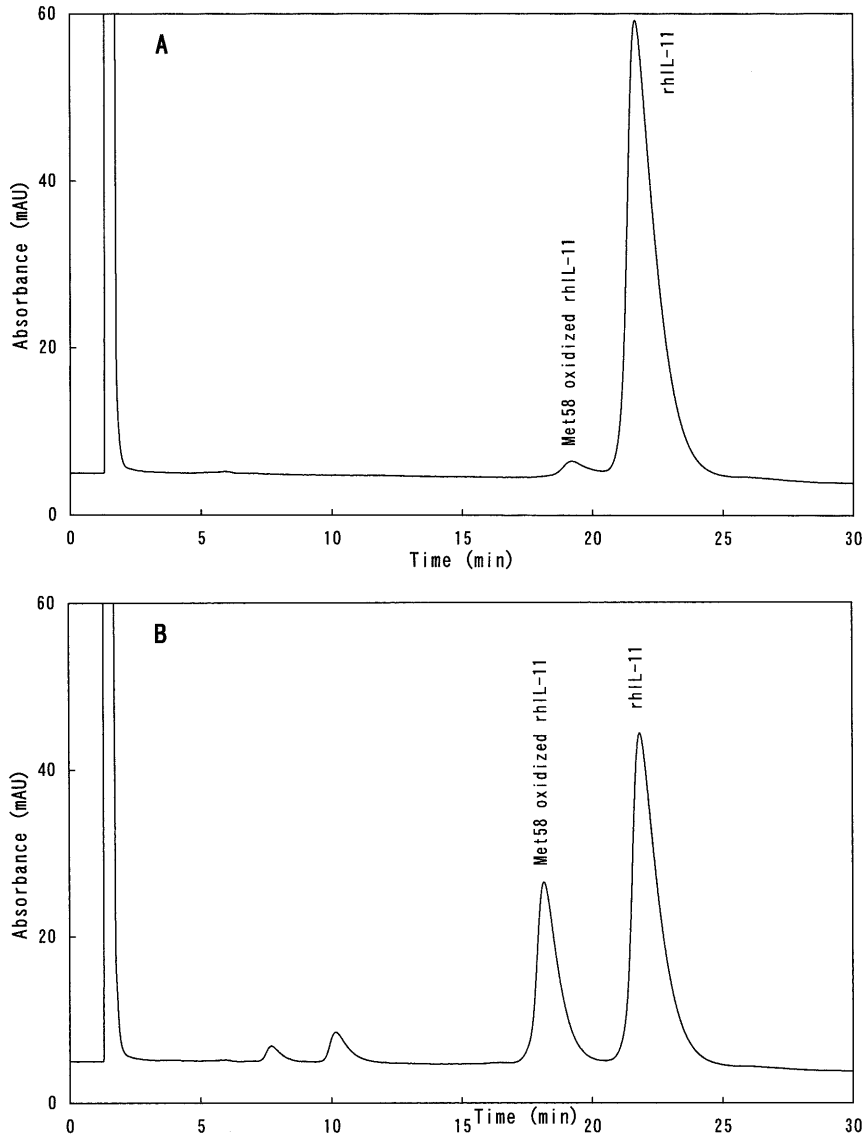

Fig. 2. Representative chromatograms of (A) rhIL-11 sample and (B) H_2O_2 treated rhIL-11.

elutes just before rhIL-11 on RP-HPLC (Fig. 2A) is Met^{58} oxidized rhIL-11. The two minor peaks in the chromatogram of H_2O_2 treated rhIL-11 (Fig. 1B) are probably Met^{122} oxidized rhIL-11, and Met^{58} and Met^{122} di-oxidized rhIL-11 [11], which can be readily separated from native rhIL-11 using a standard gradient system (data not shown).

3.2. Accuracy, precision and linearity

The fractionated Met^{58} oxidized rhIL-11 was spiked into rhIL-11 sample at five different concentrations ranging from 0.5 to 3.8%, and analyzed with this method. The results are summarized in Table 1. The recovery, measured as percent of mean content divided by nominal

content, ranged from 85.1 to 98.4%. The linear regression analysis of the oxidized form content (y) versus the nominal one (x) in the range 0.5–3.8% showed that the y-intercept, the slope and the correlation coefficient were -0.029 ± 0.070, 0.971 ± 0.030, and 0.994, respectively. The limit of detection was 0.24 μg ml^{-1} (0.3%) of the oxidized form, judging from signal to noise ratio (3:1). The R.S.D. (%) obtained using non-spiked rhIL-11 sample under five different conditions, randomizing for days, columns, instruments and analysts, ranged from 3.8 to 8.7%.

3.3. Enhancement of rhIL-11 oxidation by plastic tubes

Occasionally, increases in Met^{58} oxidized rhIL-11 have been observed during development of this

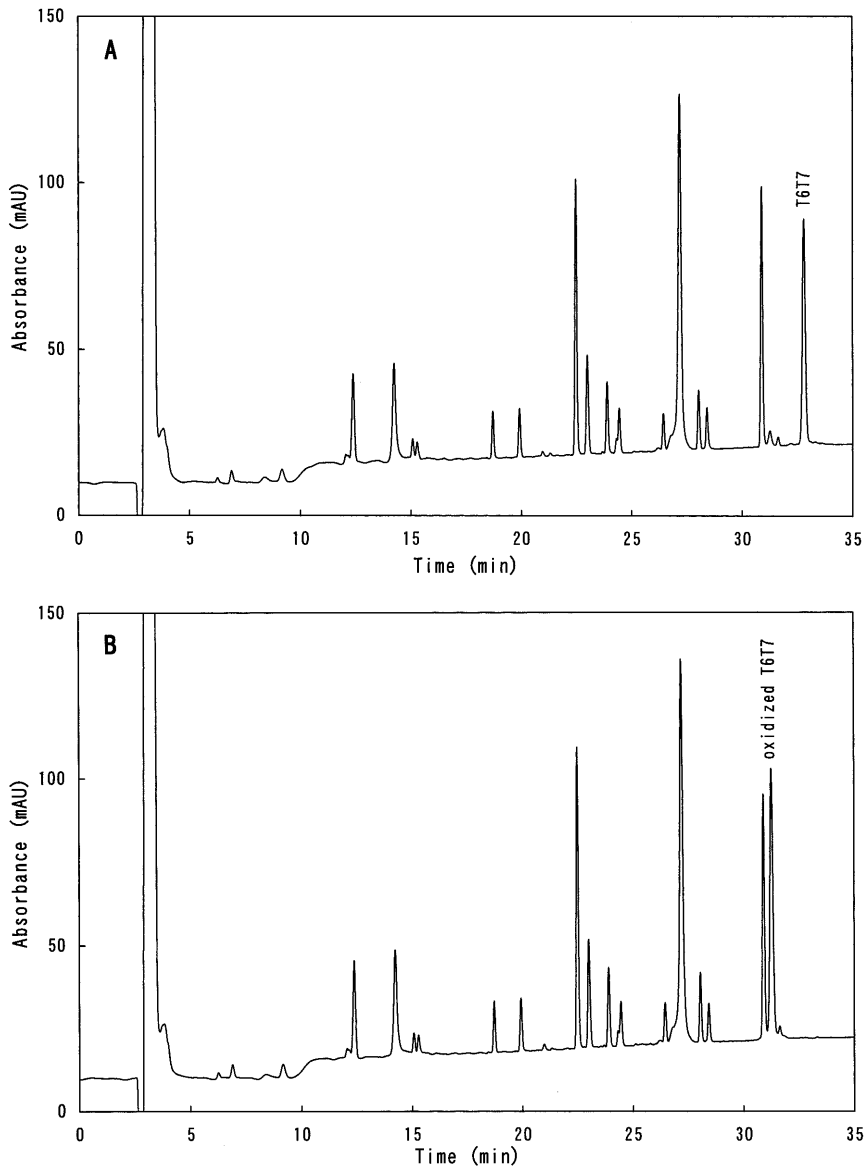

Fig. 3. Trypsin peptide mapping of (A) unoxidized rhIL-11 sample and (B) fractionated oxidized rhIL-11.

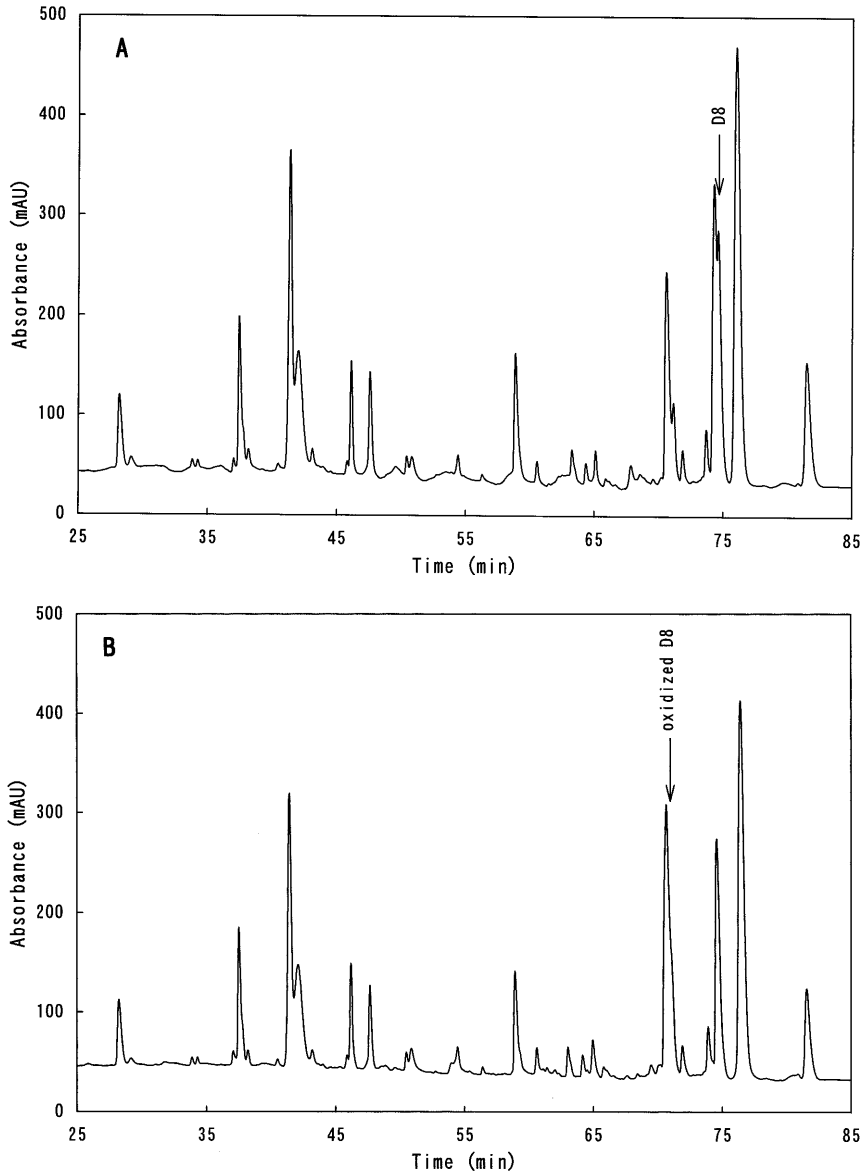

Fig. 4. Asp-N peptide mapping of (A) unoxidized rhIL-11 sample and (B) fractionated oxidized rhIL-11.

method. Disposable laboratory plastic tubes were suspected as the cause of this phenomenon. To examine the effect of plastic tubes for rhIL-11 oxidation, rhIL-11 samples were stored at 4 or 37°C in the dark in either polypropylene or polystyrene tubes. Before adding the rhIL-11 samples, the tubes were exposed to light or kept in the dark at room temperature for 25 days.

The amount of Met^{58} oxidized rhIL-11 increased during storage in all plastic tubes at 37°C (Table 2), although it remained constant during storage at 4°C (data not shown). The increase of oxidized rhIL-11 in samples stored in polypropylene tubes was higher than that in samples stored in polystyrene tubes. Additionally, Met^{58} oxidation was greatly enhanced when samples were

Table 1
Accuracy data for Met[58] oxidized rhIL-11 analysis[a]

	Spiked oxidized form (%)				
	0.5	1.0	2.0	2.9	3.8
Recovery (%)	92.5	117.2	109.1	96.8	98.8
	84.4	80.8	102.5	95.4	94.9
	78.4	86.9	83.6	94.4	94.4
Mean (%)	85.1	95.0	98.4	95.5	96.0

[a] The oxidized rhIL-11 fractionated from H_2O_2 treated rhIL-11 sample, was spiked into rhIL-11 sample at the level of 0.5–3.8%.

stored in polypropylene tubes exposed to light before rhIL-11 sample storage. Exposure to a daylight fluorescent lamp caused greater oxidation of rhIL-11 than exposure to a cool white fluorescent lamp. This result indicates that exposure of polypropylene tubes to light, especially ultraviolet light, enhances oxidation of rhIL-11. As Table 2 indicates, about two to three times more oxidation occurred in tube D than in tube C, indicating the source of the tubes may greatly influence extent of oxidation. Indeed, the increase in Met[58] oxidized rhIL-11 ranged from 2 to 33% when samples were stored in eight other polypropylene tubes obtained from various manufacturers, which were not exposed to light, at 37°C for 14 days (data not shown). Washing polypropylene tubes with water or the phosphate buffer before use revealed little effect to decrease the oxidation, suggesting that surface-bound substances are mainly responsible for this reaction (data not shown). Additionally, the increase in oxidized rhIL-11 in samples stored in light-exposed polystyrene tube B was higher than that in samples stored in no light-exposed polystyrene tube B, suggesting a possibility that exposure of polystyrene tubes to light also may enhance oxidation of rhIL-11.

Disposable plastic tubes are widely used in protein research. However, the results of this study indicate that plastic tubes, especially polypropylene ones, can cause protein oxidation. Furthermore, exposure of plastic tubes to light enhances the oxidation, especially in the case of polypropylene tubes. Plasticware is known to contain various components [13,14], which may cause oxidation of rhIL-11. Therefore, attention should be paid for selection of suitable plastic tubes used for storage of protein samples and for protection of the plastic tubes themselves from extended exposure to light while in storage.

Table 2
Enhancement of Met[58] oxidation by plastic tubes[a]

Tube			Met[58] oxidized form (%)			
Number	Material	Light	0 days	3 days	7 days	14 days
A	PS	None	2.4	3.0	3.9	5.8
B	PS	None		2.9	3.7	5.9
C	PP	None		4.4	5.7	7.2
D	PP	None		6.1	8.1	13.3
A	PS	FL	2.4	3.0	3.7	5.0
B	PS	FL		3.2	3.8	6.5
C	PP	FL		4.6	5.6	7.6
D	PP	FL		6.7	11.2	25.4
A	PS	UV-FL	2.4	2.7	3.6	5.1
B	PS	UV-FL		3.5	4.3	7.4
C	PP	UV-FL		4.2	5.9	8.7
D	PP	UV-FL		7.5	13.0	33.0
E	Glass	None	2.4	2.6	3.6	4.9

[a] PS, polystyrene; PP, polypropylene; FL, cool white fluorescent lamp; UV-FL, artificial daylight fluorescent lamp; polystyrene tubes A and B were obtained from different manufacturers. Polypropylene tubes C and D were obtained from different manufacturers.

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