# REACTIVE OXYGEN SPECIES (ROS) ASSAY TO EXAMINE PHOTOREACTIVITY OF CHEMICALS

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## 1. INTRODUCTION

The purpose of this document is to recommended protocol for reactive oxygen species (ROS) assay to examine photoreactivity of chemicals. Photoreactivity is defined as the property of chemicals that react with another molecule in consequence of photon absorption. Excitation of molecules by light can lead to generation of ROS including superoxide anion (SA) and singlet oxygen (SO) through energy transfer mechanisms. ROS assay is not a phototoxicity test but a physicochemical test similar to measurement of UV absorbance.

## 2. INITIAL CONSIDERATION

Validation studies conducted by JaCVAM showed that ROS assay has 100% sensitivity for predicting phototoxicants but a low specificity [1-3]. Based on the results of the validation studies, conducting this assay would classify a test chemical into one of 3 criterion; “Non-photoreactive”, “Weakly photoreactive” or “Photoreactive”. A “Non-photoreactive” result in this assay could predict as a non-phototoxic chemical, however, further examination on phototoxicity is necessary for “Weakly photoreactive” or “Photoreactive” result. When precipitation is found microscopically at 20 μM (final concentration), it is inadvisable to evaluate the chemical. It is inadvisable to test chemicals exhibiting peak absorbance at 440 or 560 nm, which might interfere with the ROS assay.

## 3. PRINCIPLE OF THE TEST METHOD

Drug-induced photoirritation can be defined as an inflammatory reaction of the skin after topical or systemic administration of pharmaceutical substances. Several classes of drugs including antibacterials, thiazide diuretics, non-steroidal anti-inflammatory drugs, quinolones, and tricyclic antidepressants, even though nontoxic by themselves, may become reactive under exposure to environmental light, leading to undesired side effects. The primary event in any photosensitization process is the absorption of photons of the appropriate wavelength, which allows chromophore to reach an excited state. The excitation energy is often transferred to oxygen molecules, followed by generation of ROS: SA through type I reaction and SO through type II reaction by photo-excited drug molecules. These appear to be the principal intermediate species in the phototoxic response. From the standpoint of risk assessment, previous study demonstrated that determination of ROS from pharmaceutical substances irradiated with UVA/B and visible light would be of help in recognizing their phototoxic potential.

In the ROS assay, the SO generation was detected by spectrophotometric measurement of *p*-nitrosodimethyl aniline (RNO) bleaching, followed by decreased absorbance of RNO at 440 nm [4]. Although SO does not react chemically with RNO, the RNO bleaching is a consequence of SO capture by the imidazole ring, resulting in the formation of a trans-annular peroxide intermediate capable of inducing the bleaching of RNO as follows;

SO+ Imidazole → [Peroxide intermediate] → Oxidized imidazole

[Peroxide intermediate] + RNO → RNO + Products

The SA generation could be determined by the reduction of nitroblue tetrazolium (NBT) as indicated below; NBT can be reduced by SA via a one-electron transfer reaction, yielding partially reduced (2 $e^-$) monoformazan ($NBT^+$) as a stable intermediate [5]. Thus, SA can reduce NBT to $NBT^+$, whose formation can be monitored spectrophotometrically at 560 nm.

$SA + NBT \rightarrow O_2 + NBT^+$

## 4. DESCRIPTION OF THE TEST METHOD

<u>Technical equipment</u>

- Solar simulator: e.g. Suntest CP series (Atlas Material Technology, Chicago, IL, USA) or SXL-2500V2 (Seric, Tokyo, Japan) with a fan and UVC cut filter (spectrum are shown in Appendix 1)
- UVA detector: e.g. #0037 (Dr. Hönle, München, German) or UD series (Topcon, Tokyo, Japan)
- Quartz reaction container (Ozawa Science, Aichi, Japan, Appendix 2) or its equivalent

- Microplate spectrophotometer, equipped with 440 and 560 nm filters
- Microscope
- Thermometer
- Vortex mixer
- Plate shaker
- Sonicator
- Pipettes
- Polypropylene tubes
- Plastic 96-well plates (clear, non-treat flat-bottom)
- Plastic- and glassware

## Solar simulator

Appropriate solar simulator irradiating UV and visible light should be used. The irradiation power distribution should be close to that of outdoor daylight by using an appropriate UVC cut filter. Recommended solar simulators and UVA intensity on the plate position measured by UVA detector #0037 (Dr. Hönle) are shown as follows;

- Suntest CPS+ or CPS (Atlas) with UV cut filter (<290 nm): 1.8 to 2.2 mW/cm$^2$ (e.g. the indicator setting value of 250 W/m$^2$ for CPS+) for 1 hour; 6.5 to 7.9 J/cm$^2$ of UVA intensity (Appendix 1)
- SXL-2500V2 (Seric) with UV cut filter (<300 nm): 3.0 to 5.0 mW/cm$^2$ for 1 hour; 11 to 18 J/cm$^2$ of UVA intensity (Appendix 1)

The solar simulator should be equipped with appropriate temperature control unit or fan to stabilize the temperature during irradiation because ROS production is influenced by temperature. When the solar simulator has a temperature control unit, the temperature will be adjusted at 25°C. Acceptable temperature range is 20 to 29˚C during irradiation. When a different solar simulator is used, the reference chemical set listed in section 6 should be tested prior to the test. Values of singlet oxygen (SO) and superoxide anion (SA) should be close to the values mentioned in section 6.

## Quarts reaction container

A quarts reaction container is used to avoid the loss of UV by passing through a plastic lid and vaporizing of reaction mixture [6]. The made-to-order container (Appendix 2), or its equivalent is recommended. If a different container is used, a lid or seal with high UV transmittance must be used. In this case, the feasibility study should be conducted using the reference chemicals to determine appropriate condition of UV/visible light exposure.

## Reagents

The following reagents will be used and stored according to the instructions of manufacturers.

- $NaH_2PO_4 \cdot 2H_2O$ (CAS No. 13472-35-0)
- $Na_2HPO_4 \cdot 12H_2O$ (CAS No. 10039-32-4)
- p-Nitrosodimethylaniline (RNO, CAS No. 138-89-6)
- Imidazole (CAS No. 288-32-4)
- Nitroblue tetrazolium chloride (NBT, CAS No. 298-83-9)
- Purified water

## Preparation of reagents

All reagents should be sonicated and used within 1 month after preparation. Representative preparation methods are shown as follows;

- 20 mM sodium phosphate buffer (NaPB), pH 7.4

  Weigh 593 mg of $NaH_2PO_4 \cdot 2H_2O$ and 5.8 g of $Na_2HPO_4 \cdot 12H_2O$, add 900 mL of purified water, adjust with HCl to a pH of 7.4, dilute with purified water up to 1 L, and mix.

  Stored in a refrigerator or at room temperature.
- 0.2 mM p-Nitrosodimethylaniline (RNO)

  Dissolve 3 mg of RNO in 100 mL of 20 mM NaPB.

  Stored in a refrigerator and protect from light.
- 20 mM imidazole

  Dissolve 13.6 mg of imidazole in 10 mL of 20 mM NaPB.

  Dilute the $2\times10^{-2}$ M imidazole solution 100 times with 20 mM NaPB.

  Stored in a refrigerator and protect from light.
- 0.4 mM nitroblue tetrazolium chloride (NBT)

  Dissolve 32.7 mg of NBT in 100 mL of 20 mM NaPB.

  Stored in a refrigerator and protect from light.

## Test chemicals

The test chemical should be stored appropriately until termination of the study and its stability during the test period should be confirmed. Two concentration levels, 20 and 200 μM (final concentration), should be used.

## Preparation of test chemicals

The test chemicals will be prepared using DMSO just before use. Each test chemical will be weighed in a tube, and added DMSO at the concentration 10 mM at first. The tube will be mixed with vortex mixer and sonicated for 5 to 10 min under UV-cut illumination or shade. All

preparations should be kept to protect from light. The final concentration in a reaction mixture will be set at 200 μM. When precipitation is observed at 20 μM in the reaction mixture under a microscope, 1 mM of the test chemical solution should be prepared using DMSO. In the case of DMSO-insoluble chemical, the final concentration in the reaction mixture including 20 μL of DMSO will be used at the maximum concentration without precipitation (20 or 200 μM).

## Positive and negative control chemicals

Qunine HCl (CAS No. 6119-47-7) should be used at 200 μM (final concentration) as a positive control. Sulisobenzone (CAS No. 4065-45-6) should be used at 200 μM (final concentration) as a negative control.

## Preparation of positive and negative control chemicals

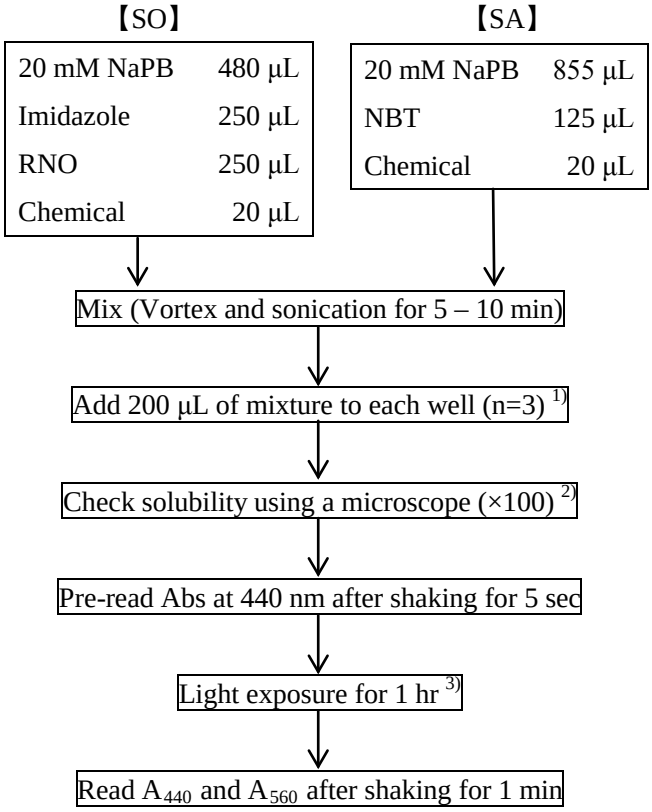
Stock solutions of qunine and sulisobenzone will be prepared at 10 mM each in DMSO (the final concentration of 200 μM) according to the above procedure, divided into some tubes, and stored in a freezer (generally below -20˚C) for up to 1 month. The stock solution will be thawed just before the experiment and used within the day.

## Solvent

DMSO (analytical grade) should be used at first. In the case of DMSO-insoluble chemical, 20 mM NaPB should be used. When a test chemical is insoluble in either DMSO or 20 mM NaPB, bovine serum albumin (BSA) or other solvent might be helpful [7-8]. Prior to use of BSA or other solvent, a feasibility study (see section 6) should be conducted using the reference chemicals to determine appropriate test conditions. However, classification for regulatory purposes should not be based on a ROS assay using BSA or other solvent until these solvents have been properly evaluated.

## Test procedure

A tube (e.g. 1.5 mL of micro tube) and a plastic clear flat bottomed 96-well microplate will be used. The reaction mixture should be prepared by vortex mixing and/or sonication under UV-cut illumination or shade. The same volume of DMSO, 20μL, will be added in a blank instead of test chemical solution.




Notes

1) It should be avoided using peripheral wells. More than one test chemical could be tested on a plate. An example of 96-well plate configuration is shown as follows;

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | | | | Singlet oxygen | | | | | | |
| B | | B | P | N | T1 L | T1 H | T2 L | T2 H | T3 L | T3 H | | |
| C | | B | P | N | T1 L | T1 H | T2 L | T2 H | T3 L | T3 H | | |
| D | | B | P | N | T1 L | T1 H | T2 L | T2 H | T3 L | T3 H | | |
| E | | B | P | N | T1 L | T1 H | T2 L | T2 H | T3 L | T3 H | | |
| F | | B | P | N | T1 L | T1 H | T2 L | T2 H | T3 L | T3 H | | |
| G | | B | P | N | T1 L | T1 H | T2 L | T2 H | T3 L | T3 H | | |
| H | | | | | | Superoxide anion | | | | | | |

B: Blank (DMSO)
P: Quinine, 200 μM
N: Sulisobenzone, 200 μM
T1-T3: Test chemical No. 1-3
L: 20 μM
H: 200 μM

2) Some chemicals may precipitate in the reaction mixture. Therefore it is important to check the solubility before the irradiation. The solubility of each reaction mixture in a well should be observed by using a microscope before irradiation. Test chemicals concentrations should be selected so as to avoid precipitation or cloudy solutions.

3) The 96-well plate will be placed in to the Quartz reaction container. A quarts cover will be set on the plate and fasten with bolts. The solar simulator and the temperature control unit (or its equivalent) will be used under a stable condition. Before and after irradiation, UVA intensity and temperature at the plate position will be measured using a UVA detector and thermometer.

## 5. DATA AND REPORTING

### Data analysis

Data from three wells for each chemical concentration will be used to calculate mean and standard deviation.

- SO

  Decrease of $A_{440} \times 1000 = [A_{440}(-) - A_{440}(+) - (a - b)] \times 1000$

  $A_{440}(-)$: Absorbance before light exposure at 440 nm

  $A_{440}(+)$: Absorbance after light exposure at 440 nm

  a: Blank before light exposure (mean)

  b: Blank after exposure (mean)

- SA

  Increase of $A_{440} \times 1000 = [A_{560}(+) - A_{560}(-) - (b - a)] \times 1000$

  $A_{560}(-)$: Absorbance before light exposure at 560 nm

  $A_{560}(+)$: Absorbance after light exposure at 560 nm

  a: Blank before light exposure (mean)

  b: Blank after exposure (mean)

### Criteria for data acceptance

The following criteria should be satisfied in each experiment.

- No precipitation of test chemical in the reaction mixture before light exposure.
- No technical problems when collecting data set (including temperature within prescribed range).
- Raw OD value: 0.02 to 1.5
- Historical positive and negative control values should be developed by each laboratory based

on a mean +/-2 SD. The following range was defined based on the 95% confidence interval (mean +/- 1.96SD) obtained from the validation data. When other solar simulators are used, establish modified criteria based on 95% confidence interval.

Positive control value at 200 μM (mean of 3 wells)

- SO: 319 to 583
- SA: 193 to 385

Negative control value at 200 μM (mean of 3 wells)

- SO: -9 to 11
- SA: -20 to 2

## Judgment criteria

Each test chemical will be judged as follows;

| Judgment[1)] | Concentration judged | SO (mean of 3 wells) | | SA (mean of 3 wells) |
|---|---|---|---|---|
| Photoreactive | 20 and/or 200 μM[2)] | ≥25 | and | ≥70 |
| | | <25 and/or P[3)] | and | ≥70 |
| | | ≥25 | and | <70 and/or P |
| Weakly photoreactive | 20 and 200 μM[2)] | <25 | and | ≥20, <70 |
| Non-photoreactive | 20 and 200 μM[2)] | <25 | and | <70 |
| Inconclusive | The results do not meet the above-mentioned criterion.[4)] | | | |

Notes

1) It can be judged based on results of one experiment because the ROS assay shows good reproducibility in the validation studies.
2) It would be judged at 20 μM only when precipitation is observed at 200 μM.
3) Precipitation before irradiation.
4) When precipitation is observed at 20 and 200 μM before irradiation, the compound is regarded incompatible with the ROS assay.

## Data quality

For a regulatory purpose, the study should be conducted under high-quality standards with data collection records readily available, preferably in compliance with GLP/GMP regulations and all documents should be checked by the Quality Assurance Unit of the laboratory.

## Test report

The test report must include the following information:

- Test chemical

  - Name and lot No.
  - Physical nature and purity
  - Storage condition
  - Stability during the test period
  - UV/vis absorption spectrum, maximum molar extinction coefficient at 290 to 700 nm, and/or photostability, if known
  - Preparation of test chemical solution
  - Final concentrations tested
- Control chemicals
  - Name, manufacturer, and lot No.
  - Physical nature and purity
  - Storage condition
  - Preparation of control chemical solutions
  - Final concentrations tested
- Solvent
  - Name, manufacturer, and lot No.
  - Justification for choice of solvent
- Irradiation condition
  - Manufacturer and type of the solar simulator used
  - Rationale for selection of the solar simulator used
  - UVA detector used
  - UVA irradiance, expressed in $mW/cm^2$
  - UVA dose, expressed in $J/cm^2$
  - Temperature before and after irradiation
- ROS assay procedure
- Acceptance and decision criteria
- Results
- Discussion
- Conclusions

## Archives

The study report and all raw data will be retained according to the SOP in the testing facility.

## 6. REFERENCE CHEMICALS FOR THE FEASIBILITY STUDY

For establishing ROS assay, irradiation condition to satisfy the recommended criteria should be determined using the positive and negative controls at 200 μM. The reference chemicals should be tested at 200 μM in a feasibility study prior to test. The reference chemicals should be selected from the following 21 chemicals judged at 200 μM in the validation studies. Recommended reference chemical set and acceptable range are shown in Table 1 and Table 2. Values of SO and SA should be close to the values.

- Photoreactive chemicals

  Acridine, acridine hydrochloride, chlorpromazine hydrochloride, diclofenac, doxycycline hydrochloride, furosemide, ketoprofen, 6-methylcoumarine, 8-methoxy psoralen (8-MOP), nalidixic acid, nalidixic sodium salt, norfloxacin, ofloxacin, omeprazole, promethazine hydrochloride, and tetracycline.

- Non-photoreactive chemicals

  Aspirin, benzocaine, camphor sulfonic acid, erythromycin, and p-aminobenzoic acid (PABA).

Table 1 Recommended chemical set for solar simulators used in the validation studies and the acceptable range at 200 μM: 3 photoreactive chemicals showing strong (No. 11), moderate (No. 12), and weak (No. 13) responses and 3 non-photoreactive chemicals (No. 14-16)

| No. | Chemical | CAS No. | SO | SA |
|---|---|---|---|---|
| *Photoreactive chemicals* | | | | |
| 11 | Doxycycline hydrochloride | 10592-13-9 | ≥115 (115 to 429) | ≥230 (230 to 468) |
| 12 | Norfloxacin | 70458-96-7 | ≥131 (131 to 271) | ≥57 (57 to 161) |
| 13 | 8-MOP | 298-81-7 | ≥31 (31 to 137) | ≥20 (0 to 126) |
| *Non-photoreactive chemicals* | | | | |
| 14 | Benzocaine | 94-09-7 | <25 (7 to 9) | <20 (7 to 17) |
| 15 | Erythromycin | 114-07-8 | <25 (-15 to 11) | <20 (9 to 19) |
| 16 | PABA | 150-13-0 | <25 (-8 to 12) | <20 (-11 to 7) |

DMSO should be used for preparation of the chemicals.

The values in parenthesis were calculated as mean +/- 1.96 SD from the validation data.

Table 2 Recommended chemical set for the other solar simulators and the acceptable range at 200 μM: 11 photoreactive chemicals (No. 21-31) and 3 non-photoreactive chemicals (No. 32-34)

| No. | Chemical | CAS No. | SO | SA |
|---|---|---|---|---|
| *Photoreactive chemicals* | | | | |
| 21 | Acridine | 260-94-6 | ≥182 (182 to 328) | ≥121 (121 to 243) |
| 22 | Chlorpromazine hydrochloride | 69-09-0 | Usually <25 | ≥66 (66 to 106) |
| 23 | Diclofenac | 15307-79-6 | ≥34 (34 to 416) | ≥47 (47 to 437) |
| 24 | Doxycycline hydrochloride | 10592-13-9 | ≥115 (115 to 429) | ≥230 (230 to 468) |
| 25 | Furosemide | 54-31-9 | ≥31 (31 to 225) | ≥20 (-7 to 109) |
| 26 | Ketoprofen | 22071-15-4 | ≥120 (120 to 346) | ≥77 (77 to 151) |
| 27 | 8-MOP | 298-81-7 | ≥31 (31 to 137) | >20 (0 to 126) |
| 28 | Nalidixic acid | 389-08-2 | ≥54 (54 to 246) | ≥88 (88 to 470) |
| 29 | Norfloxacin | 70458-96-7 | ≥131 (131 to 271) | ≥57 (57 to 161) |
| 30 | Omeprazole | 73590-58-6 | Usually <25 | ≥30 (30 to 216) |
| 31 | Promethazine hydrochloride | 58-33-3 | ≥25 (20 to 168) | ≥20 (-3 to 77) |
| *Non-photoreactive chemicals* | | | | |
| 32 | Benzocaine | 94-09-7 | <25 (-7 to 9) | <20 (-7 to 17) |
| 33 | Erythromycin | 114-07-8 | <25 (-15 to 11) | <20 (-9 to 19) |
| 34 | PABA | 150-13-0 | <25 (-8 to 12) | <20 (-11 to 7) |

DMSO should be used for preparation of the chemicals.

The values in parenthesis were calculated as mean +/- 1.96 SD from the validation data.

## 7. GLOSSARY

**ROS:** Reactive Oxygen Species, including superoxide anion (SA) and singlet oxygen (SO).

**3T3 NRU-PT:** *In vitro* 3T3 neutral red uptake phototoxicity test.

**Irradiance:** The intensity of UV or visible light incident on a surface, measured in $W/m^2$ or $mW/cm^2$.

**Dose of light:** The quantity [= intensity × time (seconds)] of UV or visible light incident on a surface, expressed in $J/m^2$ or $J/cm^2$.

**MEC:** Molar Extinction Coefficient (also called molar absorptivity) is a constant for any given molecule under a specific set of conditions (e.g., solvent, temperature, and wavelength) and reflects the efficiency with which a molecule can absorb a photon (typically expressed as $L\ mol^{-1}\ cm^{-1}$).

**Photoreactivity:** The property of chemicals that react with another molecule as a consequence of absorption of photons.

**Phototoxicity:** An acute light-induced tissue response to a photoreactive chemical.

**UVA:** Ultraviolet light A (wavelengths between 320 and 400 nm).

**UVB:** Ultraviolet light B (wavelengths between 290 and 320 nm).

**UVC:** Ultraviolet light B (wavelengths between 190 and 290 nm).

## 8. REFFERENCES

[1] ROS assay validation management team. Validation report for the international validation study on ROS (Reactive Oxygen Species) assay as a test evaluating phototoxic potential of chemicals (Atlas Suntest version), 2013.

[2] ROS assay validation management team. Validation report for the international validation study on ROS (Reactive Oxygen Species) assay as a test evaluating phototoxic potential of chemicals (Seric version), 2013.

[3] Onoue S, Hosoi K, Wakuri S, Iwase Y, Yamamoto T, Matsuoka N, Nakamura K, Toda T, Takagi H, Osaki N, Matsumoto Y, Kawakami S, Seto Y, Kato M, Yamada S, Ohno Y, Kojima H. Establishment and intra-/inter-laboratory validation of a standard protocol of reactive oxygen species assay for chemical photosafety evaluation. J Appl Toxicol, Article first published online, 13 June, 2012 (DOI: 10.1002/jat.2776).

[4] I. Kraljić, S. El Mohsni. A new method for the detection of singlet oxygen in aqueous solutions. Photochem Photobiol, 28, 577-81, 1978.

[5] Pathak MA, Joshi PC. Production of active oxygen species ($^1O_2$ and $O_2^{-\cdot}$) by psoralens and ultraviolet radiation (320-400 nm). Biochim Biophys Acta, 798, 115-26, 1984.

[6] Onoue S, Igarashi N, Yamada S, Tsuda Y. High-throughput reactive oxygen species (ROS) assay: an enabling technology for screening the phototoxic potential of pharmaceutical substances. J Pharm Biomed Anal, 46, 187-93, 2008.

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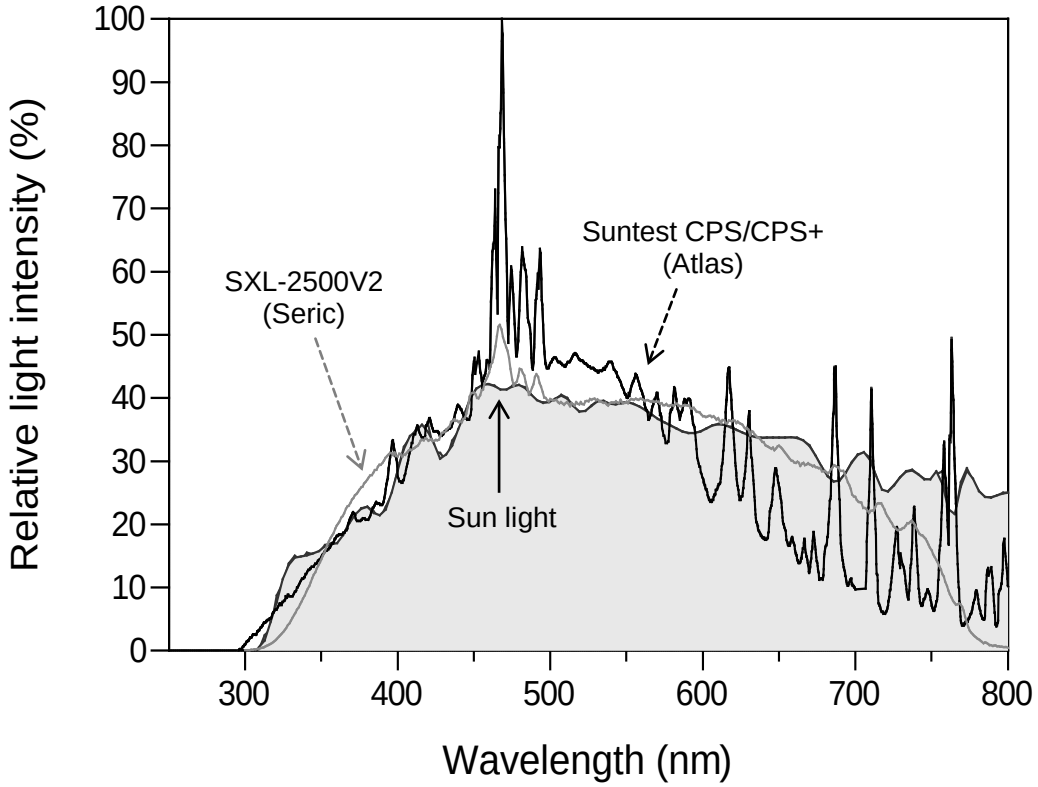
[8] Onoue S, Kato M, Yamada S. Development of an albuminous reactive oxygen species assay for photosafety evaluation under experimental biomimetic conditions. J Appl Toxicol, Article first published online, 28 Jan, 2013 (DOI: 10.1002/jat.2846).

Appendix 1 Spectrum of solar simulators used in the validation studies



Appendix 2 Quarts reaction container used in the validation studies