Evaluation of Complex Compounds in the UV-Vis Absorption Spectra Method

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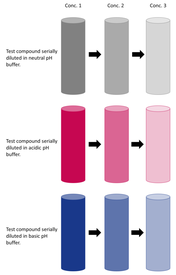
Introduction

The ultraviolet-visible (UV-Vis) spectral analysis is one of the first steps in photosafety assessments to determine the absorption of test compounds. Absorption within the range of UV and visible light (290-700 nm) that is considered significant, as defined by of a molar extinction coefficient (MEC) >1000 L $mol^{-1}cm^{-1}$, triggers potential photosafety considerations. Determination of the absorption spectra and MEC, when possible, is described in OECD Test Guideline (TG) 101: *UV-VIS Absorption Spectra*. Adopted in 1981, TG 101 provides guidance on evaluation of compounds with defined molecular weight that are soluble in water or methanol, and analysis of absorbance with a cuvette.

The growing use of botanicals, extracts, and complex mixtures without defined molecular weights in various industries calls for expanding upon the methodologies described in OECD TG 101. With the limited solvents described in TG 101 (water and methanol), novel chemistries needing evaluation, and solubility as a critical component of the assay, additional solvents were investigated for use. Failure to achieve full solubility can produce interference with the absorbance readings (i.e., filter effects or diminished absorbance values). Further, challenges may arise when selecting appropriate concentrations for complex mixtures to produce a reliable spectra. Guidance on suggesting significant absorbance using an absorbance threshold was presented in Nishida, *et. al*. (2015). Here we present approaches taken to adapt the OECD TG 101 UV-VIS Assay.

Experimental Design

Test compounds were prepared at multiple concentrations in three different solvent pH buffer systems (acidic, basic and neutral) (**Figure 1**), added to a quartz 96-well plate (**Figure 2**), and then absorbance (Optical Density (OD)) determined at wavelengths of 230 to 800 nm in 2 nm increments using a Tecan® Infinite M Nano+ (**Figure 3**). Spectral scans and OD values of selected peaks were analyzed using Magellan™ Tracker Software, and Molar Extinction Coefficient (MEC) values were calculated using peak absorbance and molarity. When MEC values could not be determined, peak absorbance values and associated wavelength were presented.



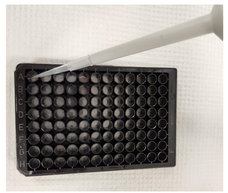


Figure 2. Loading test compound onto 96-well quartz plate.

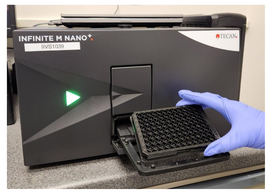


Figure 3. Infinite M Nano+ (Tecan) plate reader with 96-well loaded for absorbance determination across 230-800 nm.

Figure 1. Graphic representation of test compound preparation at 3 concentrations in three pH buffer solvent systems prior to analysis.

Evaluation of Results
Compounds with MEC values >1000 L mol^{-1} cm^{-1} are considered to have significant light absorption. For compounds without defined molecular weights, an alternative evaluation using an absorption threshold of OD ≥ 1.0, as described in Nishida, *et al*. 2015, was incorporated.

Evaluation of Solvents using Chlorpromazine
Chlorpromazine was prepared at 0.003, 0.001 and 0.0003 M in methanol, water, hexane, acetone, acetonitrile, DMSO, HBSS, ethanol, 30% methanol in water and/or 30% acetonitrile in water (all buffers pH of 7.0 ± 1.0). Spectral analysis of selected solvents are presented in **Figure 4**, with MEC values for all solvents in **Table 1.**

Determination of Weight:Volume Concentrations using p-Methoxycinnamaldehyde and Acetovanillone
Two fragrance compounds, p-methoxycinnamaldehyde and acetovanillone, were prepared at several concentrations to estimate an appropriate mg/mL concentration for evaluation using the alternate evaluation threshold. The compounds were diluted based on molarity (0.001, 0.0001 and 0.00001 M) and weight to volume (0.1, 0.01 and 0.001 mg/mL) (**Figure 5**). The MEC values were calculated for the molarity dilutions in addition to the OD peaks within the spectra of interest (**Table 2**).

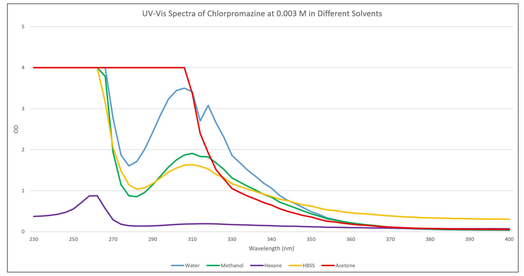
Complex Mixture Assessment using Sunscreen
A commercially available sunscreen formulation was prepared neat and serially diluted to 31.3%, 9.77%, 3.05%, 0.954%, 0.298%, 0.931%, 0.029% and 0.009% (w/v) in methanol. Absorbance within the limit of the plate reader (*e.g.*, OD < 4.0) was analyzed (**Figure 6**) to determine concentrations where a "filter" effect (e.g., producing flat lined responses with higher OD values with expected shifts in absorbance as concentrations decreased) occurred. Absorbance at 290 nm and 306 nm was determined for each concentration (**Table 3**).

IIVS would like to acknowledge the Research Institute for Fragrance Materials (RIFM) for their generous donation of the Tecan Plate Reader, as well as test compounds to "validate" the approaches and adaptions to the methodologies of this test system

Results – Chlorpromazine

Solvent	Solubility	Peak (nm)	OD at peak	MEC (L mol^{-1} cm^{-1})
Water	Soluble	307	3.40	3745.5
Methanol (MeOH)	Soluble	309	1.86	2050.9
Acetone	Insoluble	422	-0.004	-4.58
Acetonitrile (ACN)	Soluble	308	2.42	2658.2
30% MeOH in water	Soluble	306	3.29	3619.9
30% ACN in water	Soluble	306	3.10	3408.8
Hexane	Insoluble	260	0.854	939.4
HBSS	Insoluble	310	1.53	1792.5
DMSO	Soluble	308	3.07	3602.0
Ethanol	Soluble	308	2.63	3090.6

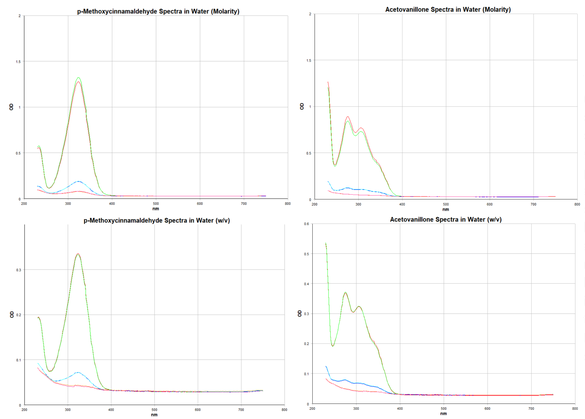
Table 1. Chlorpromazine Absorption and MEC results in 10 Solvents



Chlorpromazine, a well-known photo-reference material with MEC >1000 L $mol^{-1}cm^{-1}$ was prepared at 0.003 M in 10 different solvents. Chlorpromazine has absorption peaks ~250 nm and ~310 nm (NIST, 2021). The results of each solvent (neutral pH) are presented in **Table 1**. The peaks observed and MEC values were dependent on the solubility and solvent used. In the solvents where solubility achieved (water, methanol, acetonitrile, 30% methanol, 30% acetonitrile, and DMSO), the peaks ~306-309 nm and ~316-317 nm only vary in intensity. In acetone (insoluble) the correct peaks were not picked up and OD values were outside the readable range of the plate reader. In hexane (insoluble), the MEC calculated from peak at 318 nm does not indicate significant absorption. HBSS (insoluble) and ethanol (soluble) resulted in significant absorption but only one peak ~308-310 nm. Results showing chlorpromazine in selected solvents (**Figure 4**) are representative of how the absorption spectra can change depending on solubility and solvent.

Figure 4. Chlorpromazine absorption spectra in selected solvents water (blue line), methanol (green line), hexane (purple line) HBSS (orange line), and acetone (red line) across UV-spectra.

Results – Acetovanillone and p-Methoxycinnamaldehyde



The highest tested concentrations using molarity (0.001 M) and weight:volume (0.1 mg/mL) were evaluated in each pH buffer solvent system and compared (**Table 2**). Using either approach, the same peaks were noted for Acetovanillone and p-Methoxycinnamaldehyde, but OD values differ at 0.001 M (corresponding to ~0.2 mg/mL). Both chemicals have significant absorption (using M), but only p-Methoxycinnamaldehyde OD was >1.0 at 0.001M (~0.2 mg/mL). Collectively, these results suggest that higher concentrations should be considered when using mg/mL preparations including the evaluation with absorption threshold of OD > 1.0 by Nishida, et al. (2015). Not all concentrations will be appropriate for all complex mixtures.

Figure 5. Spectral Scan of p-Methoxycinnamaldehyde (left graphics) and Acetovanillone (right graphics) at 3 concentrations (each assayed in duplicate) using Molarity (top panels) or mg/mL (bottom panels). The top concentrations of 0.001 M and 0.1 mg/mL are represented by the green and red lines (replicates).

	Acetovanillone		p-Methoxycinnamaldehyde	
	0.001M	**0.1 mg/mL**	**0.001M**	**0.1 mg/mL**
Peak (nm)	306	306	322	322
OD	0.750	0.324	1.30	0.335
MEC (if applicable)	2642.3	NA	4574.5	NA
OD ≥ 1.0?	No	No	Yes	No
Significant absorption?	Yes	No	Yes	No

Table 2. Comparison in approach using Molarity (M) or weight: volume (w/v) of two fragrance materials

Results – Sunscreen

The OD values of replicate samples (presented as S1 and S2) of the sunscreen prepared at 9 concentrations with focus on the minimum wavelength of interest (290 nm) and the peak observed at lower concentrations (306 nm) are presented in **Table 3**. The graphics below in **Figure 6** show the spectra at select concentrations. The OD values are artificially high for 100% through 0.954%, exceeding the absorbance of the reader (*i.e.,* OD >4.0), and unable to be determined for some replicates. At concentrations ≤0.298%, a peak at 306 nm is observed. A filter effect is observed from ~400 nm to 700 nm where the OD values flat line above blank values in all concentrations.

	100%		31.3%		9.77%		3.05%		0.954%		0.298%		0.093%		0.029%		0.009%	
Conc.	**S1**	**S2**	**S1**	**S2**	**S1**	**S2**	**S1**	**S2**	**S1**	**S2**	**S1**	**S2**	**S1**	**S2**	**S1**	**S2**	**S1**	**S2**
OD @ 290	3.95	>4.0	>4.0	>4.0	3.92	>4.0	>4.0	>4.0	>4.0	>4.0	2.50	2.87	1.14	1.27	0.641	0.687	0.415	0.377
OD @ 306	3.80	>4.0	>4.0	>4.0	3.84	>4.0	>4.0	>4.0	>4.0	>4.0	3.18	3.42	1.53	1.67	0.817	0.854	0.517	0.472

Table 3. Absorption (OD) at 290 nm and 306 nm of a commercially available sunscreen at multiple concentrations

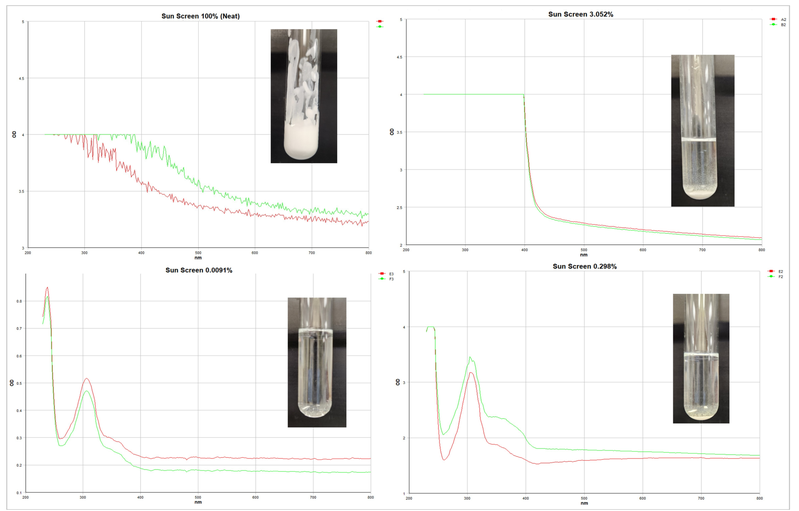


Figure 6. Spectral Scans of Sunscreen at (from top left clockwise) 100%, 3.05%, 0.298%, and 0.009% ((w/v) in methanol) of two sample replicates (red and green lines) and image of associated dilution of each concentration.

Conclusions & Future Directions

The UV-Vis assay is a crucial first step for screening of test compounds prior to evaluation in more complex and costly test systems. As the industry formulations change over time, and utilizing more complex and novel test compounds, an evolving approach using the UV-Vis assay is needed. We have further adapted the initial guidance to address throughput, materials of limited solubility, and evaluation of complex compounds.

Solubility is a critical component of this assay, as well as the solvent used. The solubility of the test compound may impact the absorbance, as well as MEC value, and ultimately the determination if additional photosafety testing is needed.

The work by Nishida, *et al.* (2015) provide an alternate approach using an absorption threshold to investigate complex mixtures and substances without defined molecular weights. A larger subset of materials covering a wider variety of industry sectors may further elucidate the target concentrations that may be needed.

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