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Article

Quantitative NMR spectroscopy reveals solvent effects in the photochemical degradation of thymoquinone

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SUMMARY

Thymoquinone, a monoterpene natural product isolated from Nigella sativa, has demonstrated potent antioxidant, cytotoxic, anticancer, and anti-inflammatory activity, making it a compound of great therapeutic potential and scientific interest. However, as a photochemically labile compound that can dimerize or decompose into other compounds upon irradiation, its clinical administration and synthetic modifications are greatly limited by its instability in the presence of light. Therefore, it is crucial to have a reliable way to quantify decreases in thymoquinone concentration as an indicator of photodegradation to provide insight into the chemical use of the compound. Here, we employed quantitative ^{1}H nuclear magnetic resonance (NMR) spectroscopy as well as computational time-dependent density functional theory (TD-DFT) calculations to identify the effect of solvation on the photochemical degradation of thymoquinone under ultraviolet light (UV). We found that the rate of photochemical degradation of thymoquinone is highly solvent dependent, and that greatest photodegradation occurs in chloroform. The results presented here are informative for future solvent selection related to the isolation and experimental usage of the compound in solution.

INTRODUCTION

Thymoquinone, a terpene commonly extracted from the seed oil of *Nigella sativa*, has been reported to have a variety of medicinal properties and can be used to treat diseases such as cancer, asthma, hypertension, and diabetes (1). Studies suggest thymoquinone exerts its biological activity by inhibiting the cyclooxygenase (COX) and lipoxygenase (LOX) enzymes and nitric oxide production to counter inflammation, lowering lipid peroxidation to boost the antioxidant defense system, and inducing cellular apoptosis in tumors (1). Although thymoquinone has highly beneficial biological characteristics and medicinal value, its chemical instability has complicated its storage and handling (2, 3).

Specifically, upon photoirradiation, thymoquinone has been found to dimerize into dithymoquinone. This reaction was first reported in the 1800s by Lallemond, *et al.*, and the structure of dithymoquinone was later verified by advances in spectroscopy (4-6). In fact, Smith and Tess demonstrated that dithymoquinone can be isolated as a single major product from the photoirradiation of thymoquinone in the solid phase (5). (**Figure 1**)

Various other reactions involving alkene 2+2 cycloadditions, wherein two pi bonds engage in a pericyclic reaction to form two sigma bonds of a cyclobutane, have been reported since the last century, many of which involve solid state chemistry and compounds in the crystal state (7-13). However, reactions in which the substrates were in solution have also been reported under both acidic and neutral conditions (14). Advances in the regio- and stereocontrol of the 2+2 cycloadditions has enabled the use of this and other similar transformations in the total synthesis of natural products including bielschowskysin, biyouyanagin, and aplydactone (15-17). However, a systematic investigation of the photodegradation behavior of thymoquinone in various solvents has not been fully reported.

In the case of thymoquinone, photodimerization is putatively driven by direct photoexcitation of one enone to the triplet state, followed by rapid 2+2 cyclodimerization with a second, ground state molecule (**Figure 2**) (5). The triplet state refers to when an electron is excited to a higher energy level and has the same spin orientation as the other unpaired electron; in contrast, the singlet excited state refers to when the excited electron retains the same spin orientation as its ground state. Alternatively, enones have been known to be photochemically excited into diradical species which can react with neighboring molecules, such as solvents, through single electron transfer radical recombination effects (20, 21). This stereospecific reaction occurs between the alkenes adjacent to the methyl group of thymoquinone in a 4-π cyclization (21). This reaction is possible through the overlap of p-orbitals of the alkenes' terminal carbons. In accordance with the Woodward-Hoffmann principles for cycloadditions, this reaction occurs via a suprafacial pathway (20, 21).

This reaction has previously been reported to occur in the presence of both visible and UV light (5, 6, 13). Irradiation with different wavelengths of light has been observed to influence the yield of the photochemical reaction, with

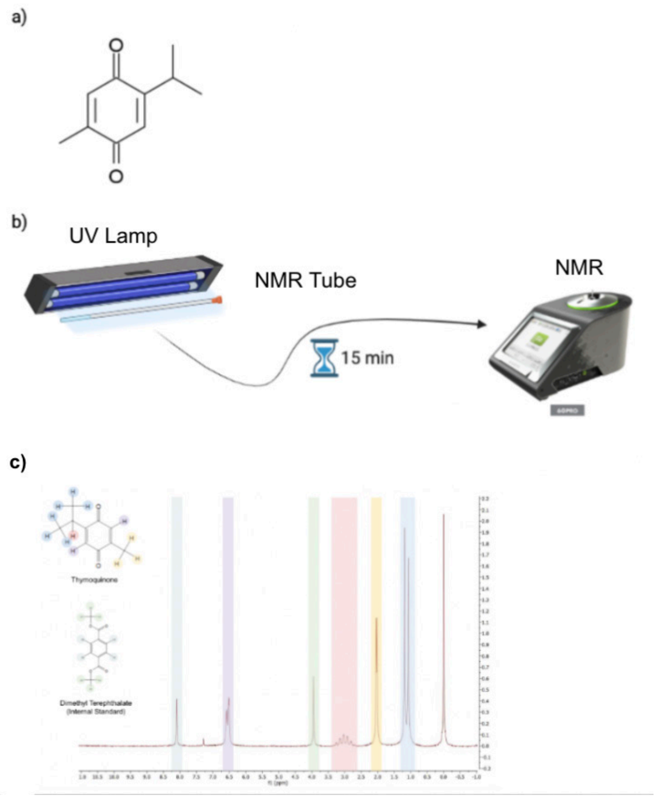

Figure 1: An overview of the experimental procedure. A) Structure of thymoquinone. B) Process by which the experiments were UV-irradiated and subsequently monitored via 1-H NMR every 15 minutes. C) NMR of thymoquinone prior to irradiation taken using Nanalysis NMReady 60PRO 60 MHz nuclear magnetic resonance spectrometer.

ultraviolet radiation generally yielding higher amounts of the dimer when compared to visible light (18, 19). However, a systematic investigation of the photodegradation behavior of thymoquinone in various solvents has not been fully reported.

We investigated possible modes of solvent-mediated suppression of the 2+2 by computational means including TD-DFT calculations to determine differences in excitation energies, as well as vibrational frequency calculations. Density Functional Theory (DFT) is a quantum mechanical method of simulating molecular structures. Because the dimerization proceeds through photoexcitation, we used TD-DFT, or time-dependent density functional theory, to model the energy gap between the excited and ground state of the orbitals of thymoquinone. This gap is also referred to as the HOMO-LUMO gap, or the energy difference between the Highest Occupied Molecular Orbital and the Lowest Unoccupied Molecular Orbital.

Recent advances in benchtop nuclear magnetic resonance (NMR) spectroscopy have enabled rapid and convenient purity analysis of small molecules as well as real time reaction monitoring capabilities (22). Here, we employed quantitative NMR spectroscopy to identify the effect of solvation in the photo-degradation of thymoquinone into dithymoquinone and other products. We hypothesized that the solvent in which thymoquinone will decompose most might be correlated with how strongly thymoquinone absorbs UV light in that solvent system. From our photo irradiation experiments, we found that thymoquinone decomposed fastest in chloroform and stayed the most stable in dimethyl sulfoxide. These results could be used in aiding others in choosing solvents in further experiments involving thymoquinone and other quinones as well as solvents that thymoquinone can be stored in and delivered biologically.

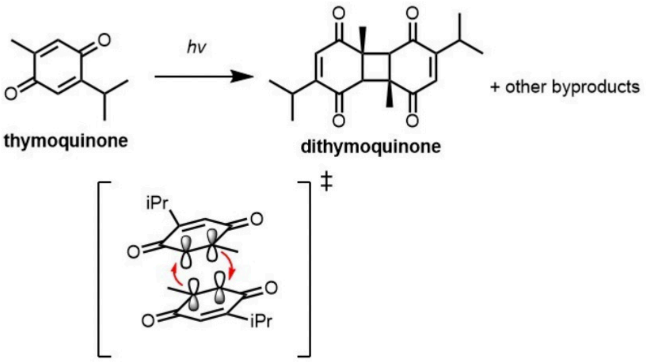

Figure 2: The dimerization of thymoquinone activated by the presence of light. Thymoquinone proceeds through a concerted 2+2 cycloaddition between an excited triplet state monomer and a ground state triplet.

RESULTS

To determine the stability of thymoquinone in different solvents, we first dissolved equal amounts of thymoquinone in the following solvents: deuterated chloroform, acetone, acetonitrile, dimethyl sulfoxide (DMSO), and methanol.

After UV irradiation with an industrial nail curing lamp of thymoquinone in the 5 solvents of deuterated chloroform, acetone, acetonitrile, dimethyl sulfoxide, and methanol, we were able to identify trends in the photo-degradation kinetics in these solvents. Stacked NMR spectra were constructed to show the visual progression of an irradiation experiment run in the deuterated solvents over the course of 255 minutes, such as DMSO (**Figure 3**).

While certain diagnostic peaks of the crude ^{1}H NMR (δ = 3.55 ppm, 1.25 ppm in chloroform) suggest the formation of dithymoquinone as one of the byproducts, the presence of other resonances after irradiation do not align with reported 1H NMR of dithymoquinone. This suggests that the mechanism of photodegradation is more complex and might be producing other discrete side products. Additional chemical shifts at 2.1 ppm are not representative of this compound, making it possible that there are multiple operative mechanisms. It is also plausible that the reaction with solvent cage molecules would lead to distinct photodegradation products such as dithymoquinone and hydroquinone.

Contrary to our hypothesis, analysis of 1H NMR shows that following irradiation with UV light in 0.25-hr (15 minute)

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intervals, thymoquinone experienced the greatest change in concentration in chloroform and the least change in concentration in DMSO (**Figure 4a**). Therefore, thymoquinone appears to be the most stable in DMSO while least stable in chloroform — in the latter, thymoquinone decomposes more than two times faster than the rate of any other solvent. Thymoquinone has the second slowest degradation rate in acetone and third in acetonitrile, leaving methanol second fastest to dimerize (**Figure 4a**). In solvents where wavelength of max absorbance is red shifted (greater than 280 nm), minimal photodegradation was observed (**Figure 4**). The absorption of light wavelengths in the 200 to 350 nm range (UV light) creates the reactive thymoquinone species, which is subject to photo-degradation and dimerization.

We also ran several time-dependent density functional theory (TD-DFT) calculations of thymoquinone when excited to the singlet and triplet states in different solvents to understand and rationalize the relative rate and mechanism by which thymoquinone decomposes over time (**Figures 5, 6**). The wavelength of maximum thymoquinone absorption was calculated from the relaxed and excited states of thymoquinone and appeared to be similar in each solvent, except for chloroform, which had a slightly lower wavelength than the others (**Figure 7**).

DISCUSSION

The percent change of thymoquinone concentration within the reaction vessel was the least in d6-DMSO, indicating that out of the solvents we studied, thymoquinone is most stable in DMSO (**Figure 4**). Thymoquinone in chloroform exhibited the greatest change in relative concentration, suggesting that the solvent pushes the reaction towards the conversion of thymoquinone into degradation products such as dithymoquinone and other unidentified product(s). Acetone, acetonitrile, and methanol had the smallest change in percent concentration after DMSO, in order of decreasing change.

We generally observed that thymoquinone underwent the slowest degradation in solvents where the compound exhibited redshifted UV-vis absorption bands in the UV-A range at 300-400 nm (DMSO, acetone) and the fastest degradation in solvents where thymoquinone exhibited blueshifted maximal UV-vis absorption in the UV-C (200-300 nm) range (chloroform, methanol, acetonitrile). We found no correlation between UV-vis maximum absorbance and solvent polarity. However, we did not take UV-vis measurements during the experimental procedure, and so cannot comment on the change in absorption spectrum as a factor of time. Interestingly, the lamp that was used for irradiation was found to not emit any detectable UV-C radiation as measured by a UV-C handheld detector. A possible justification for the observed trend was that in acetone and DMSO (315-400 nm) it is possible that the electron is excited to a non-triplet excited state (LUMO-singlet), in which case absorption of light would suppress reactivity. The dimerization reaction of enones proceeds via excitation specifically to the triplet state, so

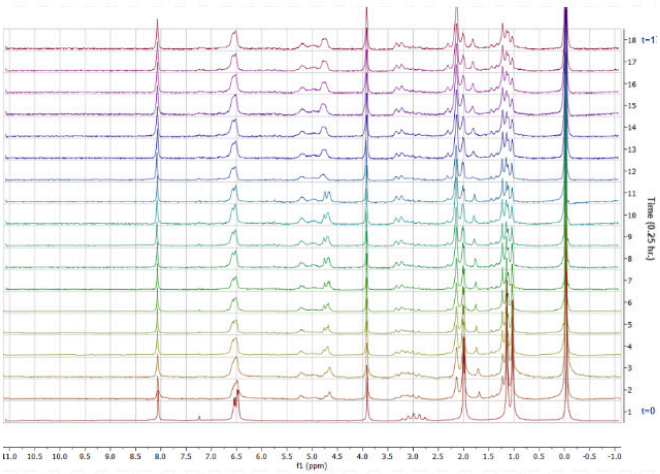


Figure 3: Time-course stacked nuclear magnetic resonance (NMR) spectra of thymoquinone photo-irradiated in deuterated chloroform. For this reaction, new peaks emerge after seventeen 15-minute intervals of constant irradiation, indicating byproducts.

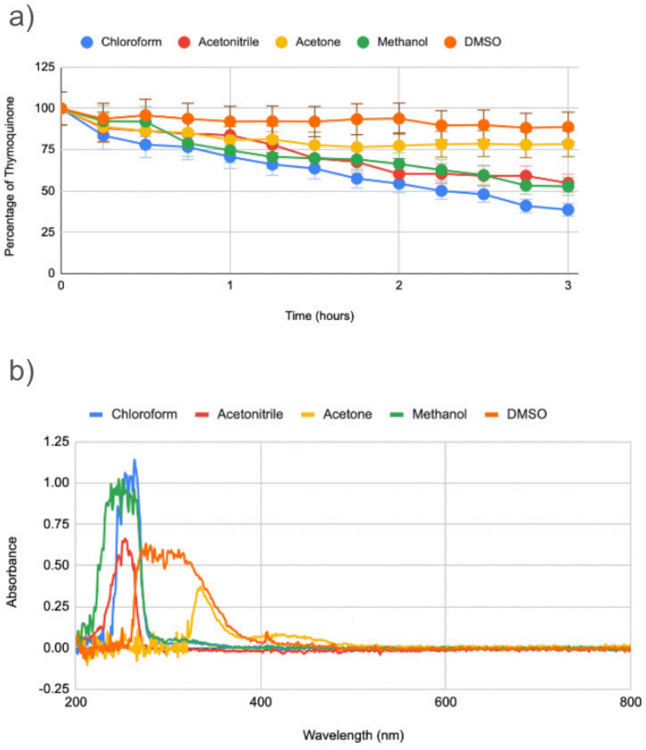
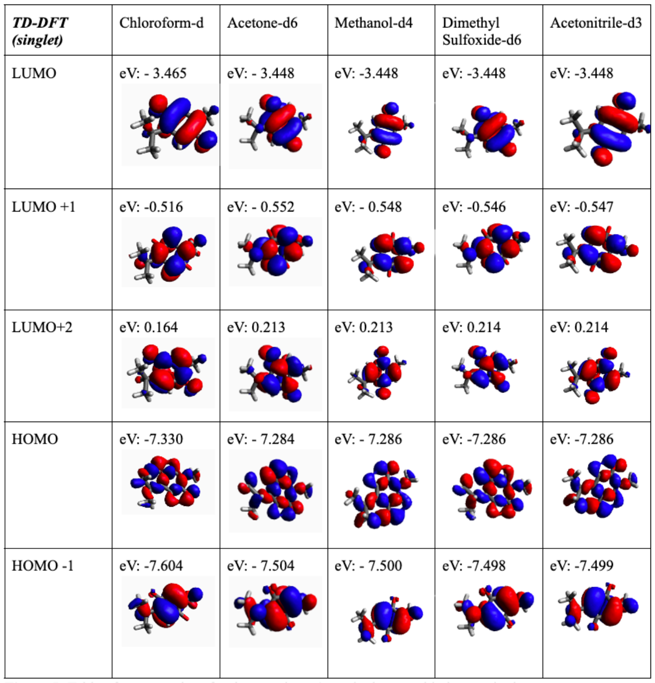
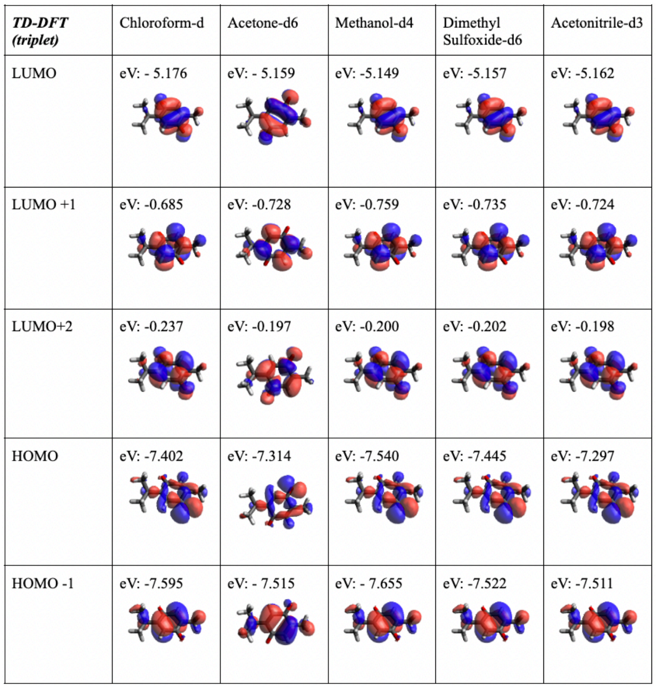


Figure 4: Graphs of thymoquinone decomposition and absorbance for each solvent. A) Thymoquinone decomposition as a function of time in various solvents via 1-H NMR spectroscopy in deuterated solvents chloroform-d, d3-acetonitrile, d6-acetone, d4-methanol, and d6-DMSO. B) Thymoquinone light absorbance in numerous solvents via UV-vis spectroscopy in deuterated solvents chloroform-d, d3-acetonitrile, d6-acetone, d4-methanol, and d6-DMSO. The spectrophotometer was blanked with the appropriate solvent prior to each measurement.



TD-DFT (singlet)	Chloroform-d	Acetone-d6	Methanol-d4	Dimethyl Sulfoxide-d6	Acetonitrile-d3
LUMO	eV: - 3.465	eV: - 3.448	eV: -3.448	eV: -3.448	eV: -3.448
LUMO +1	eV: -0.516	eV: - 0.552	eV: - 0.548	eV: -0.546	eV: -0.547
LUMO+2	eV: 0.164	eV: 0.213	eV: 0.213	eV: 0.214	eV: 0.214
HOMO	eV: -7.330	eV: - 7.284	eV: - 7.286	eV: -7.286	eV: -7.286
HOMO -1	eV: -7.604	eV: - 7.504	eV: - 7.500	eV: -7.498	eV: -7.499

Figure 5: Energy values for thymoquinone's excited state orbitals as a singlet state. Avogadro Software was used to model these TD-DFT values, which are provided in units of electron volts(eV).



TD-DFT (triplet)	Chloroform-d	Acetone-d6	Methanol-d4	Dimethyl Sulfoxide-d6	Acetonitrile-d3
LUMO	eV: - 5.176	eV: - 5.159	eV: -5.149	eV: -5.157	eV: -5.162
LUMO +1	eV: -0.685	eV: -0.728	eV: -0.759	eV: -0.735	eV: -0.724
LUMO+2	eV: -0.237	eV: -0.197	eV: -0.200	eV: -0.202	eV: -0.198
HOMO	eV: -7.402	eV: -7.314	eV: -7.540	eV: -7.445	eV: -7.297
HOMO -1	eV: -7.595	eV: - 7.515	eV: - 7.655	eV: -7.522	eV: -7.511

Figure 6: Energy values for thymoquinone's excited state orbitals as a triplet state. Avogadro Software was used to model these TD-DFT values.

excitation to LUMO-singlet would not result in dimerization. It is also possible that a confounding variable in our experiment is the presence of outside light during the measurement of NMR spectra of experiment tubes, as the instrument was not used in a dark room.

We initially used TD-DFT calculations to probe possible effects of solvation on the relative energies of excited state orbitals of thymoquinone as a potential explanation to the differences in observed reactivity. However, the electronic basis of these differences in reactivity could not be rationalized by implicit solvation TD-DFT calculations, since the energy of HOMO singlet to LUMO singlet excitation was virtually the same for all solvent systems modeled (**Figures 5, 6**); had the energy varied, it may have been possible to attribute a faster degradation of thymoquinone to a smaller excitation energy gap. It is possible that explicit solvation could have more accurately modeled the excited state electronic energies, but that is outside the scope of this study.

It is possible that a reaction with specific solvent cage molecules may lead to distinct photodegradation products. Subsequent experiments are required to identify discrete degradation products, as our past attempts were unsuccessful; additional instrumental analysis such as gas chromatography - mass spectrometry may shed light on this.

In conclusion, we utilized quantitative 1H NMR spectroscopy to identify solvent effects in the rate of photodegradation of thymoquinone and found that photoirradiation of thymoquinone in solvents in which the compound absorbed most strongly in the UV-A range (315-400 nm) resulted in relatively little decomposition, whereas the opposite was true in solvents where the compound absorbed strongly in the UV-C range (200-280 nm). Excited state computer modeling calculations by TD-DFT did not give conclusive rationalization for this observed trend, and further studies to identify the mechanistic basis for relative rates of photodegradation are currently underway. Knowledge of the solvent effect in the photodimerization and photodegradation of enones and quinones, of which thymoquinone is a model system, is useful in designing future reactions to account for degradation effects. More broadly, the photochemical stability studied here is valuable for future isolation and stability studies of thymoquinone's administration as a therapeutic agent. These results would also help to inform what solvent thymoquinone can be stored in to avoid photochemical degradation.

MATERIALS AND METHODS

Kinetic studies

To investigate the effects of ultraviolet (UV) light on thymoquinone in various solvents, 50 mg thymoquinone (AK Scientific, >98% purity by GC) was dissolved into deuterated chloroform (BTC Chemical, 99.8+% d, +0.03% v/v tetramethylsilane as an internal standard), deuterated acetone (BTC Chemical, >99.9% acetone-d6), deuterated acetonitrile (BTC Chemical, 99.8%), deuterated dimethyl sulfoxide (Millipore Sigma Magnisolv, 99.8% deuterated), and deuterated methanol (Cambridge Isotope Laboratories, 99.8%) respectively at a concentration of 0.305 mm and pipetted into five NMR tubes. Dimethyl terephthalate (AK Scientific, >95%) was added to each NMR tube at a concentration of 0.000515 mm (10 mg) as an internal standard for quantitative NMR and subsequently mixed until the internal standard was fully dissolved. The integrations of thymoquinone's proton peaks were calibrated according to the internal standard's peak, as the concentration of dimethyl terephthalate remained constant throughout the experiment. This allowed the percent thymoquinone in the reaction to be calculated as a function of time. All experiments were conducted in triplicates to increase confidence of reproducibility. The samples were then irradiated in intervals of 0.25 hrs under an industrial nail curing lamp. The nail lamp was found to emit 0 lumens of UV-C radiation using a handheld UV-C reader. 1H NMR spectra were collected on a Nanalysis NMReady 60PRO 60 MHz nuclear magnetic resonance spectrometer every 0.25 hours, for 64 scans with a relaxation period of 2 seconds per scan (**Figure 1**). UV-Vis spectra were collected on a Perkin Elmer Lambda 11 UV-visible spectrophotometer.

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Solvent	Chloroform-d	Acetone-d6	Methanol-d4	Dimethyl Sulfoxide-d6	Acetonitrile-d3
Singlet HOMO-LUMO Gap (eV)	3.865	3.836	3.838	3.838	3.838
Triplet HOMO-LUMO Gap (eV)	-2.154	-2.125	-2.135	-2.129	-2.124
Wavelength of Maximum Thymoquinone Absorption Singlet LUMO-Singlet HOMO(nm)	320.787	323.212	323.044	323.044	323.044
Wavelength of Maximum Thymoquinone Absorption Singlet HOMO-Triplet LUMO(nm)	575.600	583.455	580.722	582.358	583.730
Vibrational frequency (J)	29702.199	53325.980	15456.986	66593.580	14404.177

Figure 7: Summary of computational data for each solvent. HOMO-LUMO gaps and wavelengths of maximum thymoquinone absorption of both singlet and triplet thymoquinone, calculated via TD-DFT. The broad spectrum UV source used in experiments had a range of wavelengths listed in the table, given that it was found to emit UV radiation, not including UV-C.

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