

Excited State Dynamics in the MALDI Matrix 2,4,6 Trihydroxyacetophenone: Evidence for Triplet Pooling Charge Separation Reactions

Kristopher M. Kirmess[†], Richard Knochenmuss^{‡*}, Gary J. Blanchard[‡], Gary R.
Kinsel[†]

[†] Department of Chemistry and Biochemistry, Southern Illinois University at Carbondale,
Carbondale, IL. 62901

[‡] Tofwerk AG, Uttigenstrasse 22, 3600 Thun, Switzerland

[‡] Department of Chemistry, Michigan State University, East Lansing, MI. 48824

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RATIONALE: Excited state pooling reactions are a central part of some models of ultraviolet MALDI ionization mechanisms. Evidence has been found for pooling in several matrix materials, but a recent report of pure exponential fluorescence decay at MALDI-relevant laser fluences suggested that 2,4,6 trihydroxyacetophenone (THAP) may be an example of a matrix in which pooling does not occur (Lin et al., *Rapid Comm. Mass Spectrom.*, **28**, 77 (2014)). However, those data were instrumentally limited in dynamic range and signal/noise ratio, and the conclusion does not take into account several aspects of THAP excited state dynamics.

METHODS: Using time-correlated single photon counting, and absorption and emission spectroscopies, the excited state dynamics of THAP are reexamined.

RESULTS: Like many other aromatic ketones and acetophenone, isolated THAP molecules undergo very efficient intersystem crossing. No fluorescence is observed in dilute solution. In the solid state, efficient fluorescence reappears, but is non-exponential even at very low excitation intensity. The solvent used for sample preparation was found to have a large effect on the spectra and decay curves. Needle-like crystals seem to be correlated with reduced intersystem crossing.

CONCLUSIONS: THAP solid state fluorescence is entirely due to intermolecular interactions. Activation of fluorescence, instead of quenching, is a clear indicator of delocalized excited state phenomena in THAP. Contrary to the conclusions of Lin et al., the greatly increased singlet lifetime in the solid state substantially increases the probability that pooling-type reactions are indeed involved in ionization processes. The sensitivity of fluorescence and phosphorescence on sample morphology appears to reflect changes in intermolecular interactions due to crystal packing. Pooling charge separation pathways based on known triplet-triplet ionization reactions of aromatic ketones are proposed.

*Author for correspondence:

rknochenmuss@gmx.net

INTRODUCTION

Matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy is a widely used technique for the analysis of peptides, proteins, and bio and synthetic polymers, among others. Though used extensively, fundamental processes which occur within MALDI that result in ionization are still debated. While secondary ion-molecule reactions in the expanding ablation plume do not appear to be controversial, there is less consensus regarding primary ionization mechanisms leading to the initial charge separation.^[1]

Models for primary ionization differ substantially. The “lucky survivors” model postulates preformed ions present in the sample prior to laser ablation.^[2,3] Some randomly escape neutralization in the expanding plume. Since it makes no quantitative predictions, the model relies on qualitative consistency with a variety of test cases. Evidence has been presented that this mechanism can coexist with others.^[4]

Laser heating plays a more direct role in the polar fluid model.^[5,6] It was proposed that the matrix fluid in the early plume may be sufficiently hot and solvating to allow thermal formation of ion pairs. The polar fluid model has received little attention until recently, when it was revived by reports of much lower ionization yields than found in previous studies. If it is as low as 10^{-7} to 10^{-9} ,^[7-12] the temperature and solvation requirements are less stringent than if it is several orders of magnitude higher. However, dielectric constants are low at high temperature and in supercritical fluids, and at typical MALDI fluences plume temperatures are around 1000 K or below. Along with low ion formation rates and geminate recombination losses, it has been recently shown that the ion yield in the polar fluid model must be far below all experimental estimates.^[13]

As Ehring and Hillenkamp noted,^[14] in ultraviolet MALDI a variety of non-thermal processes become possible due to electronic excitation of the matrix. The coupled physical and chemical dynamics (CPCD) model is based on one of these; excited state pooling reactions.^[15-17] The primary ionization step is taken to be the result of concentration of energy by mobile excitons colliding. In this manner the energy barrier to ionization is directly overcome in a non-thermal process. The CPCD makes quantitative predictions that have been tested against a wide

variety of MALDI phenomena, most recently the wavelength and fluence dependence of ion yields for three matrixes^[18] as measured by Soltwisch, et al.^[19]

Independent evidence (by means other than mass spectrometry) for pooling in MALDI matrix materials has been reported for 2,5 DHB, cinnamic acid, CHCA, ferulic acid, sinapinic acid, ABA and 3-hydroxy picolinic acid.^[20-24] (The CPCD fits to the chloro-CCA data of Soltwisch are also evidence for pooling in that matrix.^[18]) Fluorescence of the solid matrix material is the most common method previously used to directly investigate pooling. It may be quenched at higher fluences if the probability of singlet exciton annihilation increases when they are created in closer proximity. Pooling then leads to non-exponential fluorescence decay. Early in the decay, exciton annihilation competes with fluorescence, but slows as exciton density drops.

Lin et al. recently used time-resolved luminescence to evaluate possible pooling in the matrix 2,4,6 trihydroxyacetophenone (THAP), and compared it to the previously studied 2,5 dihydroxybenzoic acid (DHB).^[25] With a streak camera they were able to measure decay curves at high, MALDI-relevant, excitation densities.

For DHB, the results confirmed the earlier results and analysis.^[20, 21] At MALDI fluences, singlet excitons are separated by only a few molecular diameters, so pooling is fast enough to cause a marked decrease in initial lifetime and non-exponentiality in the decay. For example, at a mean exciton separation of 10 molecules, the hopping rate in DHB of about 0.1 ps^[21] leads to a pooling time constant on the order of 100 ps, This is readily differentiated from the solid state lifetime without pooling of around 600 ps.

Lin et al. observed solid state THAP fluorescence, but were unable to detect any non-exponentiality in their decay curves, even at high fluence. This led them to suggest THAP may undergo purely thermal ionization along the lines of the polar fluid model.^[25]

However, this conclusion assumes that the matrix is intrinsically fluorescent and that pooling is revealed by quenching at higher excitation density. As we show here, in the case of THAP, that assumption is false. Instead, and in contrast to other matrixes, it is the appearance of solid state fluorescence which provides evidence for extended intermolecular interactions. Decay

curves acquired with higher signal to noise ratio and with higher dynamic range also show that fluorescence is not exponential. Finally, analogy to other hydroxy acetophenones suggests pooling charge separation reactions in the triplet states of neighboring THAP molecules in the solid state.

EXPERIMENTAL

Sample Preparation

THAP (2,4,6 trihydroxyacetophenone 98% and >99.5%) was purchased from Sigma-Aldrich USA. All solvents were of >99.9% purity. The THF was not stabilized.

Thin samples of the matrices were prepared and simultaneously purified by sublimation onto pre-cleaned 2.5x2.5 cm aluminum substrates. About 100 mg matrix was placed at the bottom of a sublimation chamber. The substrate was affixed to the bottom of a cold finger using vacuum adhesive tabs. After five minutes under reduced pressure (0.1 Torr), the cold finger was filled with cold water (10 °C) and heat was applied by a sand bath to the base of the chamber. The temperature of the sand bath was monitored throughout sublimation (180° C) and maintained below the melting point of the sample (219° C). After 20 minutes, the heat source was removed from the chamber, which remained under vacuum for an additional ten minutes, and was then vented to atmospheric pressure.

The purified matrix material was characterized by proton NMR, and FTIR spectroscopies, as well as MALDI, HPLC and thin layer chromatography. In all cases, the material appeared to be pure. As noted below and in the supplementary material, the optical absorption and emission spectra after sublimation were also consistent with increased purity of the sample. Residual impurities cannot, of course, be ruled out, but purity was apparently increased by the procedure used.

Luminescence Spectroscopy and Decay Curves

Emission spectra of the sublimed MALDI matrices were collected using a Fluorolog fluorometer (Horiba Scientific, Edison, NJ. USA). The excitation wavelength was 337 nm, excitation/emission slit widths were 2.0 nm, and the scan range 400 – 650 nm.

Fluorescence lifetime measurements were performed using a time-correlated single photon counting (TCSPC) instrument that has been described in detail previously.^[26] Briefly, the light source is a CW passively mode-locked Nd:YVO₄ laser (Spectra Physics Vanguard) that produces 2.5 W average power at both 355 nm and 532 nm, with 13 ps pulses at 80 MHz repetition rate. The 355 nm output of the source laser is used to excite a cavity-dumped dye laser

(Coherent 702) operating at 674 nm with Sulforhodamine 640 dye (Exciton) and producing 5 ps pulses at a repetition rate of 4 MHz (Gooch and Housego cavity dumping electronics). Pulses from the dye laser are divided into excitation and reference arms, with the reference channel being detected by a photodiode (Becker & Hickl PHD-400-N). The excitation pulse train is frequency doubled using a LiIO₃ Type I second harmonic generation crystal. The fundamental is separated from the second harmonic with a BG-3 color filter (transmittance <0.01 at the fundamental, >0.98 at the harmonic) and the second harmonic polarization is rotated to vertical using a quartz rhomb pair. The 337 nm average power at the sample was *ca.* 1 mW and the beam focused to a 10 μm diameter spot. This resulted in a fluence per shot of approximately 0.3 mJ/cm². This is well below the intensities for which photoelectron emission has been observed from THAP (8.4 mJ/cm²).^[27]

Emission is collected using a 40x reflecting microscope objective (Ealing) and the components polarized parallel (0°) and perpendicular (90°) with respect to the vertically polarized excitation pulse are separated using a polarizing cube beam splitter. The polarized signal components are detected simultaneously using two microchannel plate photomultipliers (MCP-PMT, Hamamatsu R3809U-50), after passing through subtractive double monochromators (Spectral Products CM-112).

Although the instrument is equipped to measure emission anisotropy, the highly structured microcrystalline samples investigated here unfortunately lead to highly variable and poorly reproducible polarization ratios. All decay transients reported here are therefore unpolarized.

The detection electronics (Becker & Hickl SPC-132) produce a *ca.* 40 ps response function for each detection channel. Data acquisition, detector bias, collection wavelength and time window are all controlled using a program written in-house in LabVIEW® (National Instruments).

Multiple decay curves were acquired on multiple locations of the samples. As a typical example, three repetitions at three locations for a total of nine decay curves. Both the replicates at a location and the measurements at different locations were highly consistent, to within the experimental uncertainty.

All micrographs were obtained using an inverted optical microscope (Leica DMIRB) equipped with a Coolsnap MYO 14-bit (Photometrics) CCD camera.

RESULTS AND DISCUSSION

In dilute solutions (10^{-6} M) negligible prompt emission was observed from sublimed THAP. A weak slow component, with a time constant of over 2 ns, was observed, as seen in Fig. 1. This is similar to the parent compound, acetophenone, for which no fluorescence, but weak phosphorescence has been observed.^[28]

Figure 1 here

This behavior is a characteristic of aromatic ketones,^[32] in which intersystem crossing from an initially excited π - π^* singlet to an n - π^* triplet is an allowed, rapid and efficient process (El-Sayed rule).^[29] After intersystem crossing, in addition to phosphorescence a variety of photochemical processes are known, often referred to as Norrish pathways types I and II. These have been observed in acetophenone and dihydroxy acetophenone.^[30, 31]

In this context, it seems evident that using fluorescence quenching as an indicator of exciton pooling in solid THAP, as in ref. [25], is based on false premises. If the isolated molecule is already nonfluorescent, pooling cannot be detected by fluorescence quenching. Any processes that take place in the triplet state also have no effect on the fluorescence.

Since THAP is intrinsically nonfluorescent, even small quantities of fluorescent impurities can generate misleading results. The decay curves reported in ref [25] were for commercial 98% purity THAP. Impurities at much lower levels than 2% have been shown to affect excitation dynamics in MALDI matrix materials.^[21] Jaskolla et al. have shown that very low concentrations of trapping impurities strongly affect the MALDI process in CHCA, in a manner consistent with pooling.^[33] As shown in the supplementary material, evidence of active impurities was found in our unpurified THAP material, affecting both the spectrum and the decay curves. Significant questions thus remain about the role of impurities in the results of ref. [25]. All results shown here are for THAP purified by sublimation.

Figure 2 shows that even after purification, solid THAP does fluoresce when excited at MALDI-relevant wavelengths. In addition to the weak phosphorescence visible after about 1.5 ns, strong fast emission is evident. This is remarkable, in view of the solution behavior. Internal conversion processes have been dramatically inhibited, not enhanced, by interaction of two or

more THAP molecules in close proximity. This behavior (fluorescence only in the solid state) has also been observed for the MALDI matrix materials ferulic acid, sinapinic acid and alpha-cyano-4-hydroxycinnamic acid.^[34]

Figure 2 here

Fig. 2 shows decay curves of three separately prepared samples, from the same bottle. In each, fluorescence gives way to phosphorescence at longer times, but the rates of both and their relative contributions vary widely. The fluorescence decay constant ranges from roughly 50 to 200 ps, a factor of four. The relative intensity of the phosphorescence varies by at least two orders of magnitude, at this observation wavelength.

Fig. 2 also shows evidence for multiple excited state processes in addition to intersystem crossing. The peaks of the decay curves are significantly offset, by more than the width of the instrument function. This offset is not correlated with the time constant of the fastest decay component. The decay in the first nanosecond is also not uniform. The initial slope is greater than at intermediate times, as shown by the dashed lines in the inset. These were empirically fit to the first few picoseconds after the peak, as a guide to the eye.

Several dynamical excited state processes are clearly active in solid THAP, but the variability in the curves of Fig. 2 is surprising. To investigate the possibility of residual impurities, individual sublimed samples were scraped off the substrate, divided into parts, each part redissolved in a different solvent, and then again dried. An example of one such experiment is shown in Fig. 3.

Figure 3 here

The subdivided sample exhibits variability similar to that observed from the separately prepared samples, although it was initially homogeneous. All of the features found in the samples of Fig. 2 are again observed in Fig. 3, at both short and long time scales. Recrystallization from different solvents apparently is affecting the intermolecular interactions which are responsible for the fluorescence, excited state dynamics and intersystem crossing. This hypothesis seems to be supported by the substantial differences in the morphology of the samples, as shown in Fig. 4.

Figure 4 here

The sample crystallized from ACN formed fine needles radiating from nucleation centers. This sample exhibited the smallest degree of intersystem crossing and phosphorescence. The initial fluorescence peak also appeared at the largest delay, but the fluorescence time constant was relatively fast.

The sample prepared from THF formed small crystallites, $<5\ \mu\text{m}$ diameter, which appeared to be roughly spherical in shape. This sample exhibited the longest fluorescence lifetime, although it peaked earlier. A substantially higher degree of intersystem crossing occurred, compared to the ACN sample.

The EtOH sample was intermediate between the other two. The particles formed were again roughly spherical, but many were of considerably larger size than in the THF sample, $\geq 10\ \mu\text{m}$ diameter. Their largest dimension remained, however, smaller than the length of the ACN needles. The decay peak is again early, the fluorescence decay similar to the ACN sample, but the intersystem crossing relatively strong.

In addition to the emission decay curves, the absorption and emission spectra of solid THAP depend on the preparation solvent, as seen in Fig. 5. Most features in the absorption spectra retain their positions, but the relative intensities of the S_1 and S_2 absorption bands change considerably. The emission spectra vary in both position and in width. Both the high and low energy tails of the bands are affected.

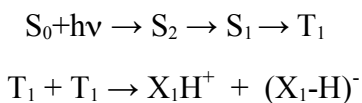
Figure 5 here

Fig. 5 shows that the typical UV MALDI wavelengths of 337 and 355 nm excite not the lowest singlet state, S_1 , but rather the S_2 . Some of the dynamics observed above may be associated with relaxation from S_2 to S_1 . This is supported by the decay curves shown in Fig. 6 for excitation at 450 nm, predominantly into the S_1 . The fast fluorescence component is still clearly present but relatively less intense. Once again the curves vary substantially as a function of solvent used to create the samples. The cause of the marked difference in S_1 and S_2 fluorescence yields is currently unclear, but it seems evident that the overall rate of intersystem crossing is higher if excited into the S_1 .

Fig. 6 here

Several factors could contribute to the variable excited states dynamics of solid THAP. Residual solvent in the dried material cannot be excluded, but since no fluorescence was observed in dilute solutions of various solvent polarities, this seems insufficient. Furthermore, the redissolved samples were reexamined by FTIR spectroscopy after preparation, and no evidence of solvent inclusion was found. More likely is differing packing of individual molecules in the crystal lattice, with consequent changes in intermolecular contact points. Since the key nonbonding lone pair atomic orbitals involved in the photodynamics of ketones are localized on the carbonyl oxygen, even small differences in packing might significantly change wavefunction overlap with neighboring molecules. Since the intermolecular interactions may extend farther than one nearest neighbor, dimensionality, anisotropy and defect density could all play a significant role. For example, it seems notable that the long needles formed in ACN result in the lowest intersystem crossing, and greatest delay in the fluorescence.

Regarding mechanisms of THAP ionization in MALDI, the well studied photochemistry of acetophenone and other aromatic ketones may provide some useful guidance. Triplet-triplet annihilation can be efficient, even at low excitation density, and is used to increase the efficiency of photovoltaic cells, among other applications. (Note that triplet reactions do not quench fluorescence, and therefore cannot be detected in that way.) The pooling process would then be similar to the singlet-only model, except that the reaction occurs not upon collision of singlet excitons, but when nearby molecules undergo intersystem crossing, or when mobile triplet excitons collide:



Where X denotes a triplet or a singlet, since either may be the product of the annihilation reaction. Excitation at 355 or 337 nm has been assumed, since these are by far the most commonly used wavelengths. Triplet-triplet reactions have been observed in numerous aromatic ketones, many reacting by annihilation to produce ions in solution after excitation at 355 nm.^[35] Furthermore, photoelectron ejection has been observed from three MALDI matrixes, including THAP, at fluences far below the ablation thresholds, after excitation at 337 nm.^[27] This is

consistent with efficient pooling reactions, and with molecular dynamic simulations of MALDI, in which ionization precedes ablation.^[36]

Regarding specific charge separation processes in THAP, excited state intramolecular proton transfer in 2-hydroxyacetophenone is known to occur due to increased acidity of the hydroxyl group and increased basicity of the carbonyl oxygen in the triplet excited state.^[37] The hydroxyl in 4-hydroxyacetophenone is also more acidic in the excited state^[38] but is obviously less able to engage in an intramolecular proton transfer, and thus is more available for an intermolecular reaction.

In the solid state, proximity of the 4-hydroxy group of one THAP to the carbonyl of another would facilitate intermolecular charge transfer, if both molecules were excited to the triplet. The following scheme may thus be considered a type of pooling reaction, when the reactants are both in the triplet state:

Scheme 1 here

Other charge separation pathways could be proposed based on intermediate diradical cleavage products, which may react further to distonic ions.

CONCLUSIONS

As is typical of aromatic ketones, acetophenone, and acetophenone derivatives, isolated THAP molecules are not fluorescent in solution due to efficient intersystem crossing. THAP becomes partially fluorescent in the solid state, a phenomenon observed in other MALDI matrix materials. The fluorescence after excitation into the S_2 , as for typical MALDI laser wavelengths, is not single-exponential. We find evidence for at least two relaxation steps from the S_2 state, before intersystem crossing. Excitation into the S_1 results in a lower ratio of fluorescence to phosphorescence, but again nonexponential decay. The singlet dynamics and the rate of intersystem crossing are strongly dependent on sample preparation, and correlate with macroscopic changes in crystal habit.

These phenomena are believed to result from interaction of the carbonyl lone pair electrons with neighboring THAP molecules. Intermolecular wavefunction overlap may, for example, mix π - π^* character from the neighbor into originally n - π^* states, making intersystem crossing less allowed. Since the relevant nonbonding orbitals are highly localized, this effect could be very sensitive to minor variations in crystal packing.

Regarding pooling in THAP, the conclusion of Lin et al. was based on a false assumption and on insufficient data quality, and is found to be incorrect. Indeed, the inverse conclusion is instead supported. Fluorescence is induced, not inhibited, by intermolecular interactions in the solid state.

In addition, pooling-type charge separation pathways in the solid state from two neighboring triplet THAP molecules are proposed, based on analogous reactions previously observed in a number of aromatic ketones. The known increases in acidity of the phenolic hydroxyl groups and increased basicity of the carbonyl in hydroxy acetophenones also suggest a possible proton transfer mechanism.

Since many MALDI matrix materials exhibit low fluorescence quantum yield, have molecular structures favoring intersystem crossing via n - π^* states, and have been observed to emit photoelectrons well below the ablation threshold, triplet pooling reactions may be widespread MALDI ionization pathways, perhaps more so than singlet mechanisms.

Figures

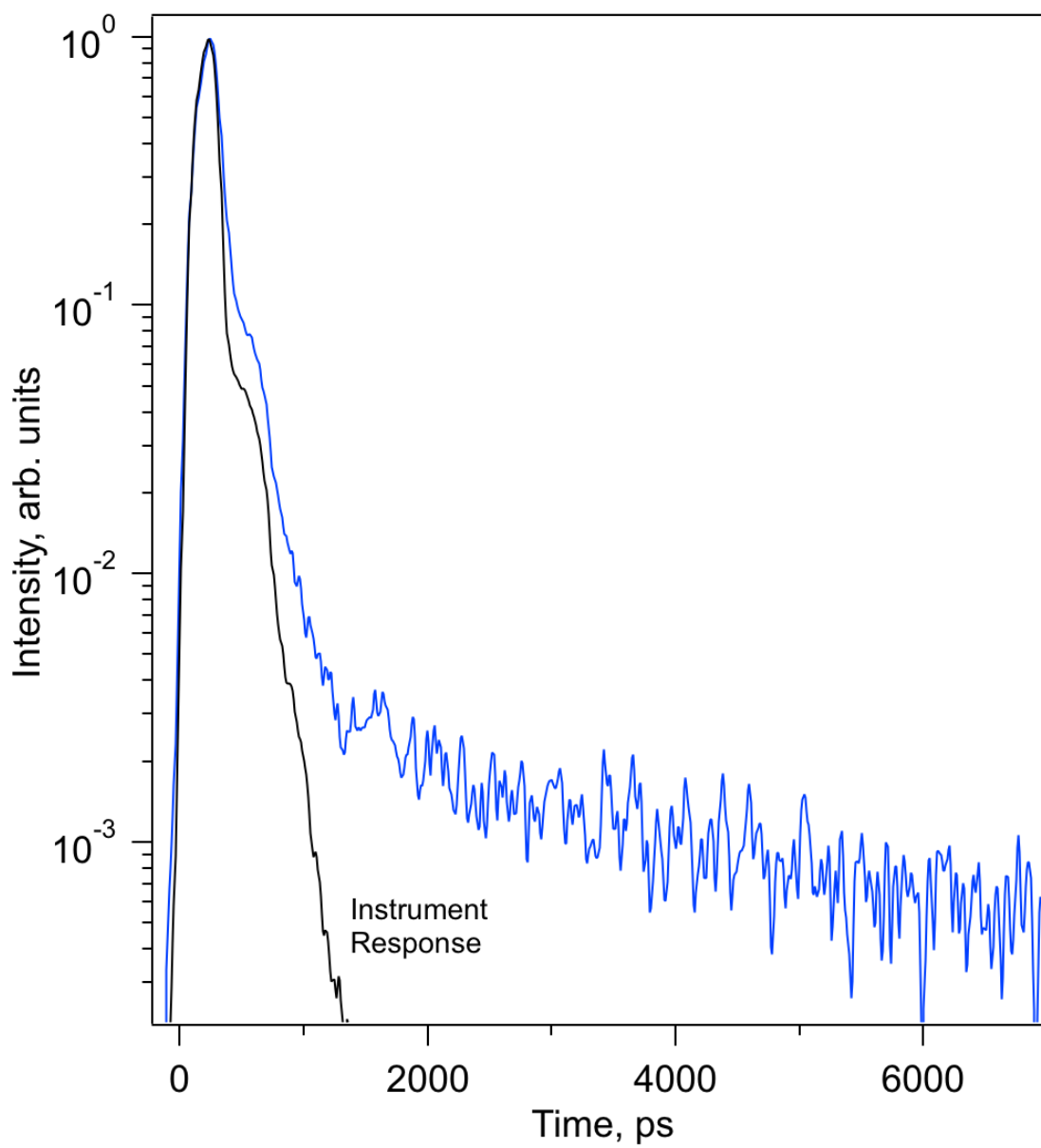


Figure 1. Fluorescence decay curve of sublimed THAP in THF solution (10^{-6} M), at room temperature. Excitation was at 337 nm, emission was observed at the peak of the band, 523 nm.

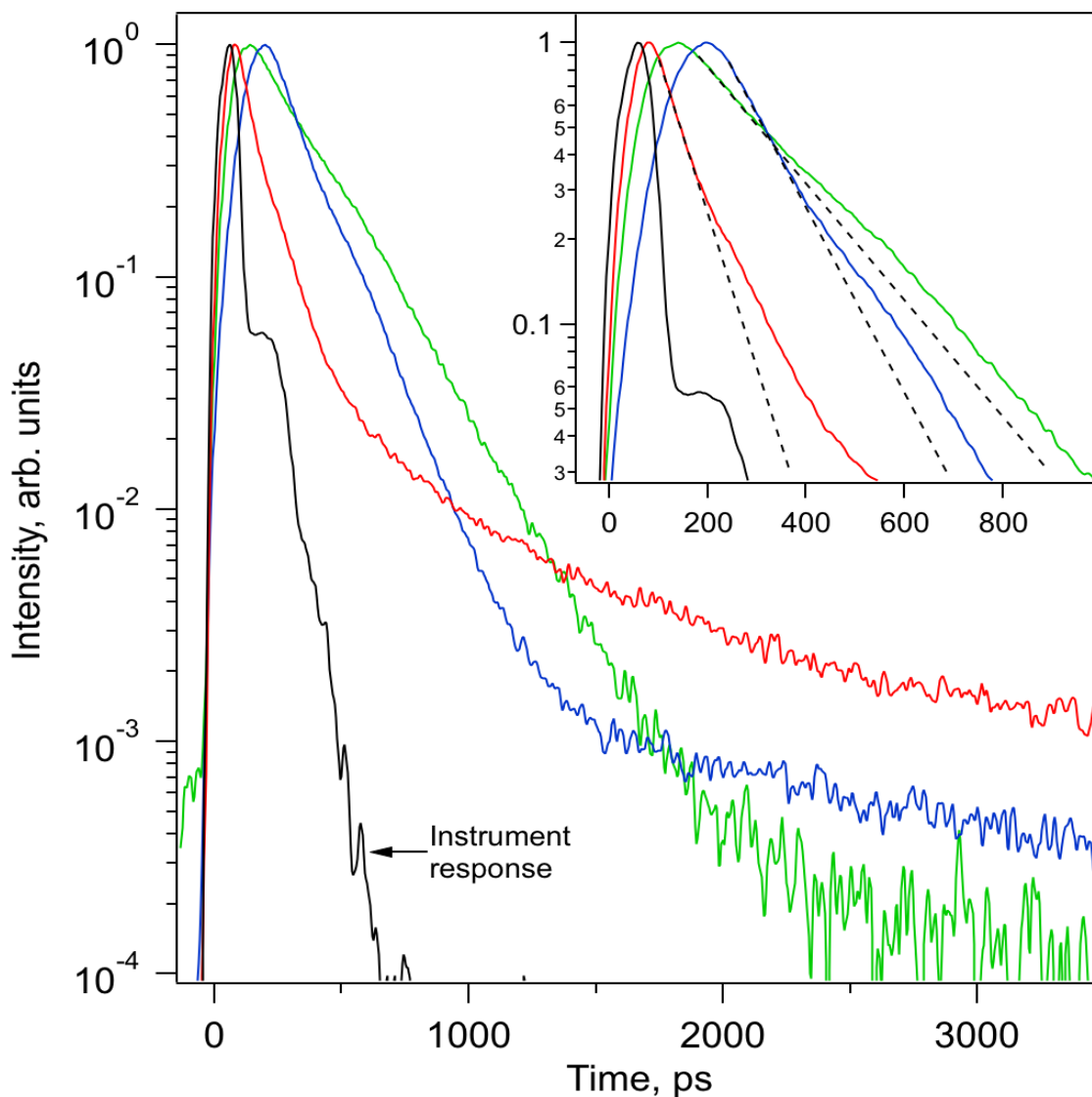


Figure 2. Fluorescence decay curves of three nominally identical sublimed THAP samples in the solid state, at room temperature. Excitation was at 337 nm, emission was observed at the peak of the band, 523 nm. Although the three samples were from the same source and prepared similarly, they exhibit substantially different decay behaviors. In all cases strong fluorescence occurs before giving way to typical $n-\pi^*$ triplet emission. The inset shows the varying peaks of the fluorescence, as well as the breaks in the slope of the emission curves (horizontal axis in picoseconds). The colors in the online version are onto differentiate the samples. See the text for further discussion.

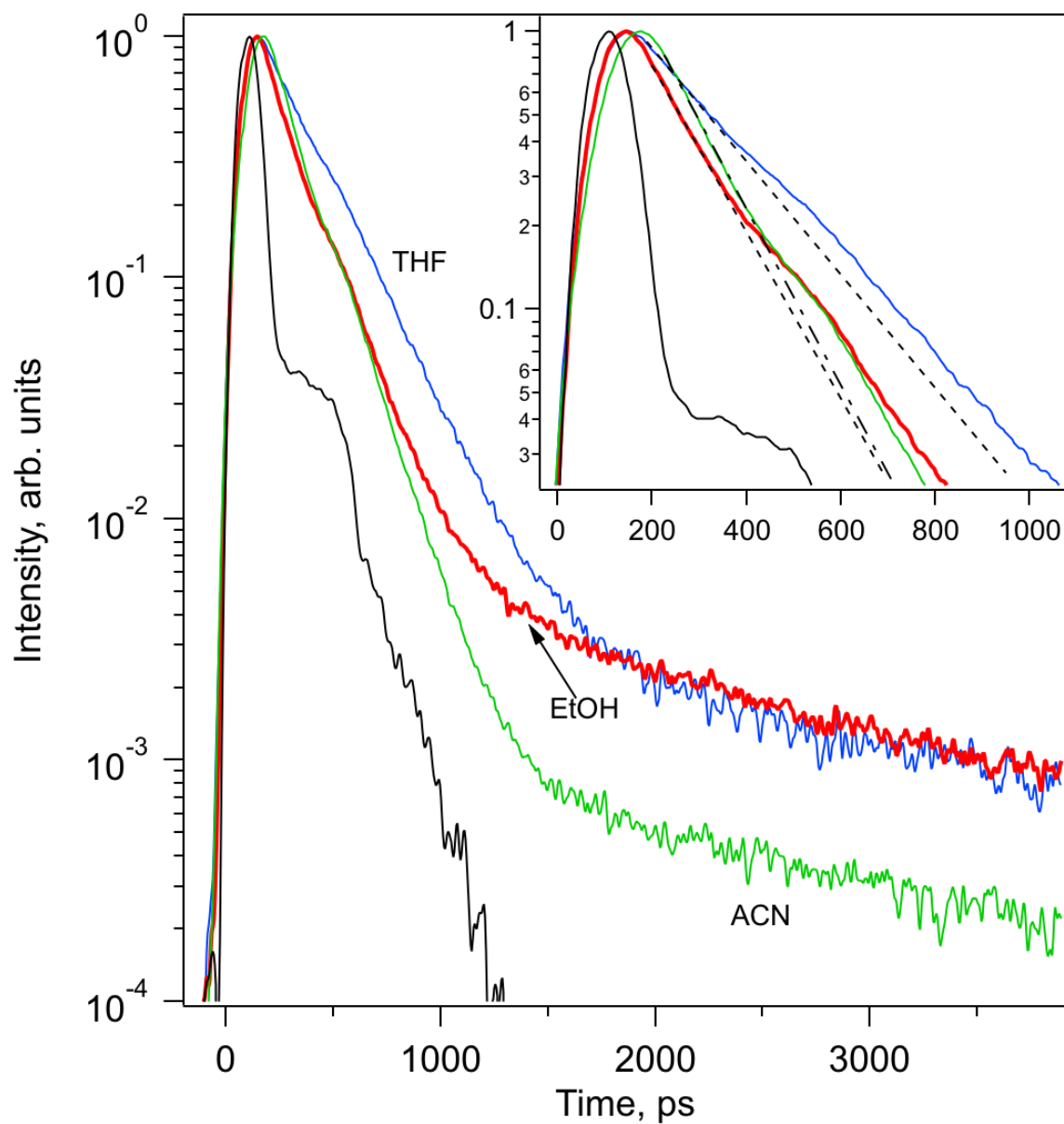


Figure 3. Fluorescence decay curves of one sublimed THAP sample in the solid state, after dissolution and drying. The solvents used were: blue THF, red EtOH, green ACN. The inset shows the varying peaks of the fluorescence, as well as the breaks in the slope of the emission curves (horizontal axis in picoseconds). See the text for further discussion. Excitation was at 337 nm, emission was observed at 523 nm.

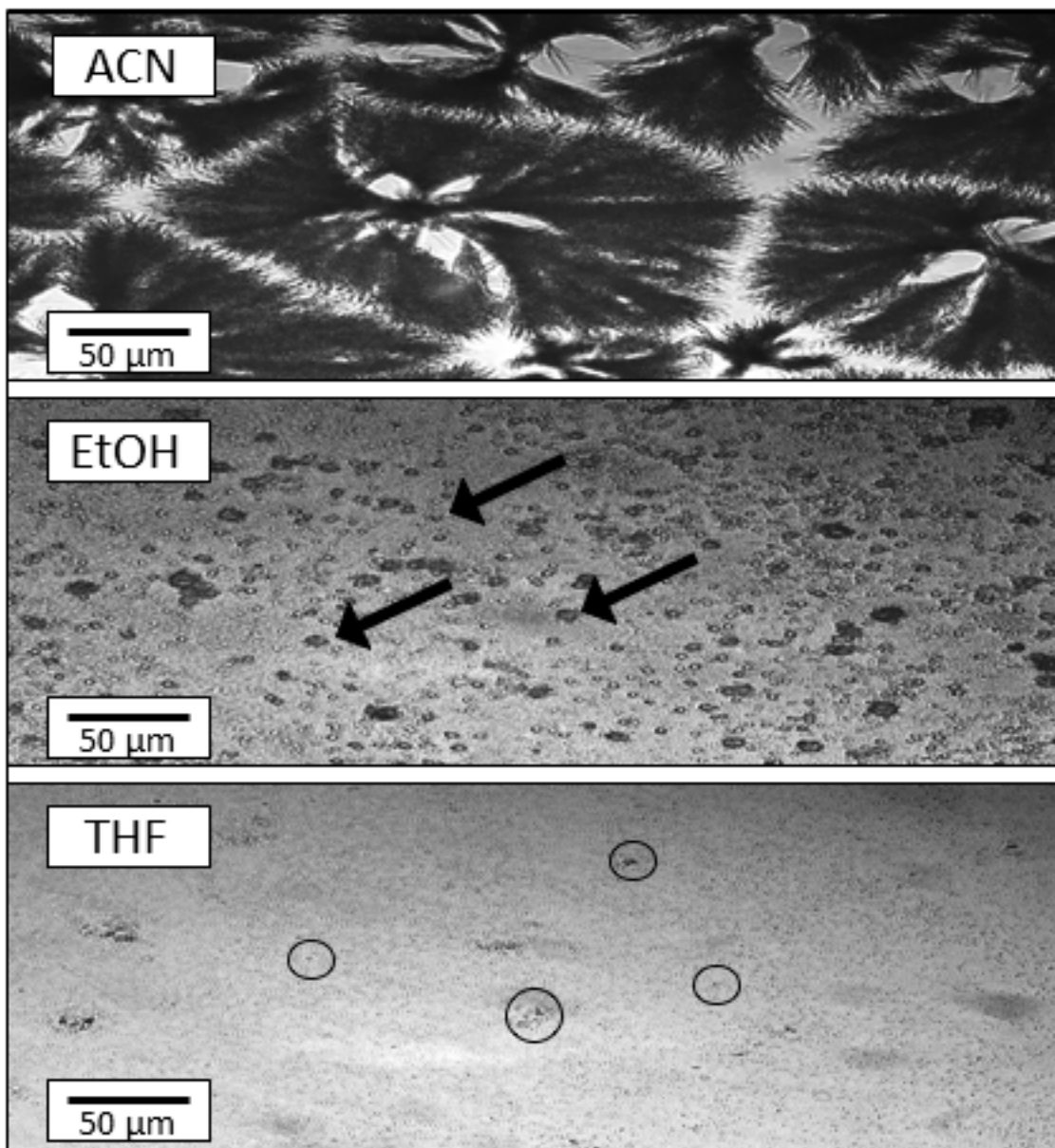


Figure 4. Micrographs of the redissolved and dried samples, corresponding to the curves in Figure 3.

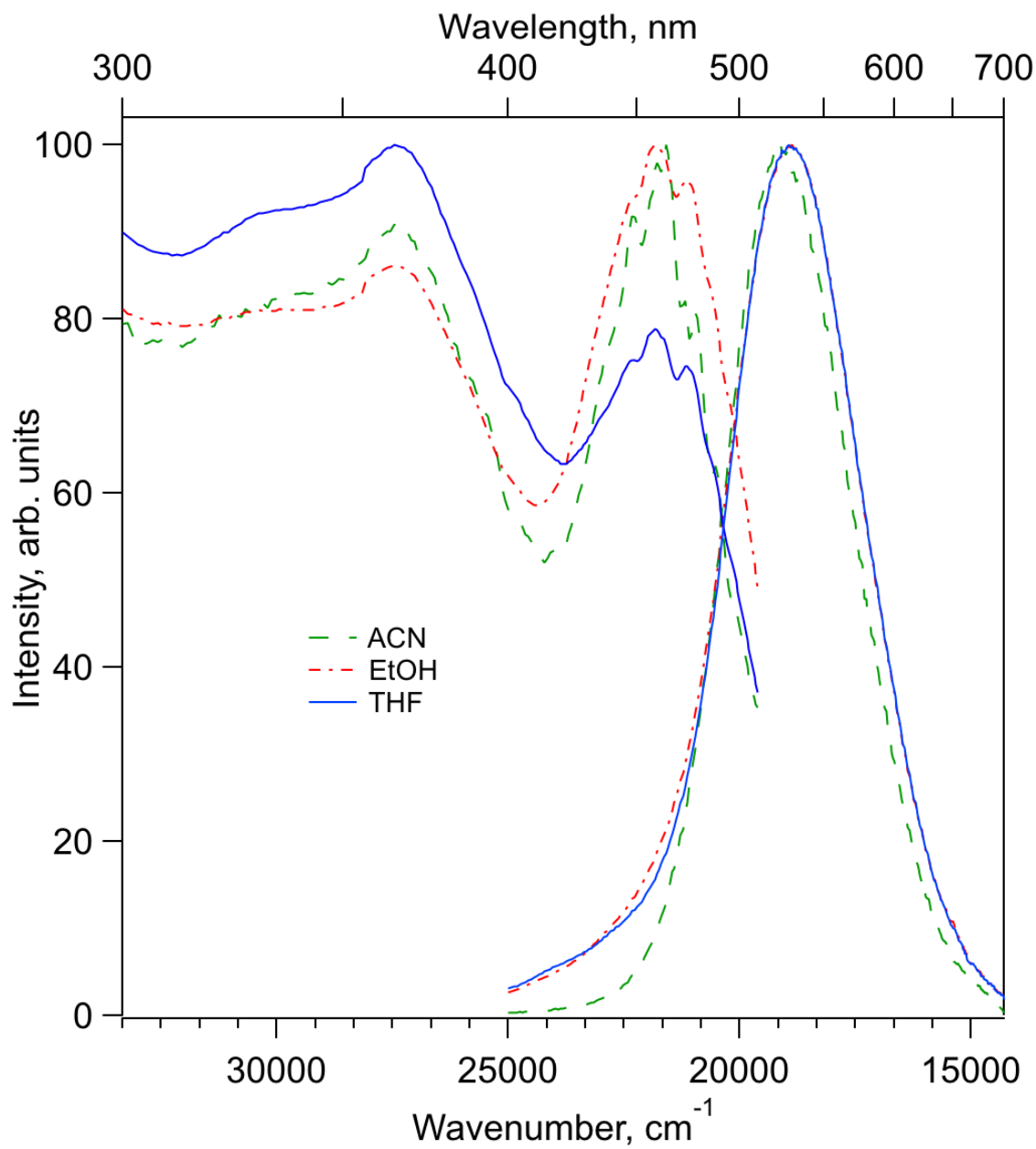


Figure 5. Absorption and emission spectra of sublimed, redissolved and dried samples, prepared from three different solvents.

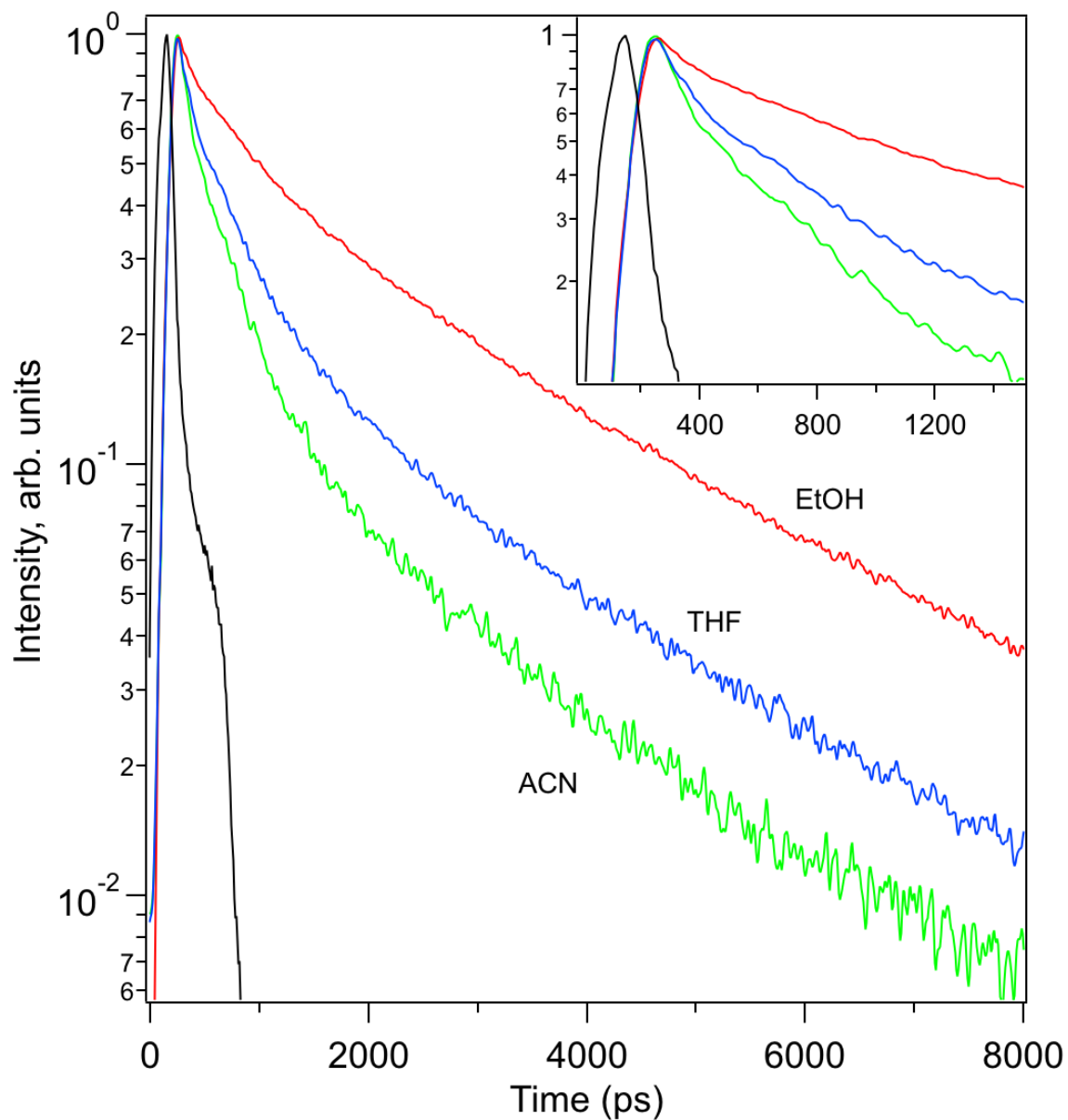
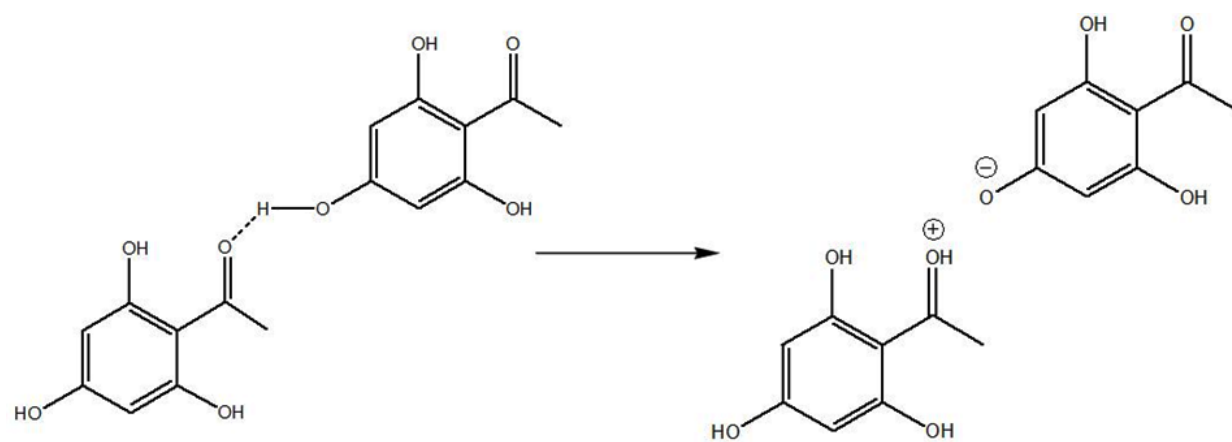


Figure 6. Fluorescence decay curves of three nominally identical sublimed THAP samples in the solid state, at room temperature. Excitation was at 450 nm, emission was observed at the peak of the band, 523 nm. Compared to excitation at 337 nm, fluorescence is weaker compared to the slower phosphorescence. The decays vary considerably as a function of preparation solvent.

Scheme 1:



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