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PAPER

Photoacoustic and luminescence characterization of nitrogen heterocyclic aromatic UV-MALDI matrices in solution†

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A group of nitrogen heterocyclic aromatic compounds used, among others, as UV-MALDI matrices was studied. By using spectroscopic, luminescence and photoacoustic techniques, as well as time resolved phosphorescence for singlet oxygen production determination, the behaviour of 9-aminoacridine (9AA), 3-aminoquinoline (3AQ), 2-(2-aminoethylamino)-5-nitropyridine (AAN) and 3,4-dihydro-7-methoxy-1-methyl-9*H*-pyrido[3,4-*b*] indole (harmaline, HLA) in acetonitrile solutions is described. The results show that for these compounds radiationless processes that release prompt heat to the media are a quite important deactivation mechanism.

Introduction

Matrix-assisted UV laser desorption–ionization is the soft ionization method suitable for the mass spectrometry analysis (UV-MALDI MS) of macromolecules including thermo-labile compounds and bio- and synthetic polymers.[1,2] The samples analyzed are solid or quasi-solid and prepared by dispersing the analyte into a large excess photosensitizer known as a matrix.[3–5] The mixture is irradiated with a laser pulse and, among other species, intact molecule analyte gas ions are formed. Although this technique has been successfully used for the last 20 years, the mechanism of ionization during MALDI is still poorly understood, and the effort to find adequate models to explain the complete process is continuously undertaken by different groups.[3–5]

It is generally accepted that the matrix provides several key features, including the absorption of the laser energy, given that the laser photons are not absorbed by the analyte, and the codesorption and ionization of the embedded analyte compounds. Various models describing the desorption–ionization processes have been proposed.[3–11] There is evidence that analyte ionization occurs during the ablation process on the surface,[12,13] as well as in the expanding plume by a collisional mechanism in the gas phase.[14,15] Both processes, in source decay during ablation and ionization in the plume, might involve the ground state and electronic excited states of the matrix.

Another model called "pooling" tries to explain the process as an "indirect photoionization" of the matrix based on the singlet–singlet annihilation of two neighbour matrix molecules in the electronic excited singlet state (S_1),[16] similar to the well described triplet–triplet annihilation process.[17] Thus, one of the matrix molecules can reach a higher excited singlet state (S_2) and can induce the photoexcitation and photoionization of the matrix. This would be like a two-photon process. The contribution of this mechanism to the MALDI of analytes is not clear although this singlet–singlet annihilation has been described as the dominant mechanism for depopulating the S_1 state in a matrix crystal (*i.e.*, 2,5-dihydroxybenzoic acid and two additional matrices) excited at typical MALDI fluences.[18]

Although it is recognized that after the photon absorption and electronic excitation of the matrix a thermal ablation occurs,[11,12,16] the photochemistry in general and particularly the radiationless deactivation of the compounds used and/or suggested as UV-MALDI matrices are not studied. As previously indicated, the knowledge of the thermal deactivation process of common UV-MALDI matrices together with other mechanistical aspects discussed in the literature can play an important role in understanding why some matrices lead to more abundant post-source decay as well as prompt decay of the analyte.[19–21]

Even though the desorption–ionization process in UV-MALDI-MS takes place in a vacuum atmosphere chamber, atmospheric pressure (AP) MALDI sources have been described recently and are very attractive because of the possibility of reducing equipment.

In this context, in a previous work, we studied the photophysical behaviour of classical UV-MALDI matrices 2,5-dihydroxybenzoic acid (gentisic acid, GA), 2,4,6-trihydroxyacetophenone (THAP), *trans*-3,5-dimethoxy-4-hydroxycinnamic acid (SA), *trans*-4-hydroxy-α-cyano-4-hydroxycinnamic acid (CHC), 9*H*-pirido[3,4-*b*]indole (nor-harmane, norHo) and 1-methyl-9*H*-pirido[3,4-*b*]indole (harmane, Ho) in acetonitrile under different atmospheres using photoacoustic techniques.[22] The compounds

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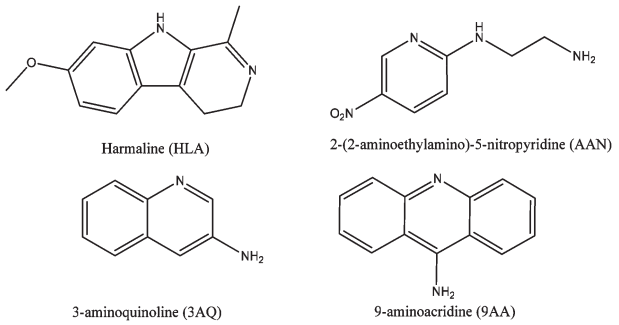

Fig. 1 Chemical structure of the UV-MALDI MS matrices studied in this work.

studied here, shown in Fig. 1, have been recently described as UV-MALDI MS matrices for the analysis of biological compounds such as lipids, proteins and carbohydrates. Thus, 2-(2-aminoethylamino)-5-nitropyridine (AAN) has been proposed as an efficient matrix for analysis of lipids in negative ion mode.[23] 9-Aminoacridine (9AA), alone or in combination with other compounds, as co-matrix, has been proposed to be used for the detection of phospholipids,[24,25] and 3-aminoquinoline (3AQ) has been used for ionization of plant carbohydrates.[26] Furthermore, harmaline (HLA) has been described as a very efficient matrix for cyclic and acyclic oligosaccharides and proteins, both in negative and in positive ion mode.[27,28]

It is noteworthy that there is not much information in literature about the luminescence and spectroscopic properties of these compounds. Among these publications, only some aspects of the photophysics of 3AQ,[29,30] 9AA[31] and more detailed studies with HLA[32–40] have been previously described. Particularly the radiationless deactivation releasing heat to the media, as well as the capability of singlet oxygen generation by oxygen quenching have not been previously studied.

For UV-MALDI experiments, matrices and analyte solutions are prepared in neutral medium (*i.e.* acetonitrile, acetonitrile–water or water for carbohydrates and glycoconjugate compounds) and in an acid medium (*i.e.* H_2O–trifluoroacetic acid 0.1% for protein analysis). After solid sample preparation, matrix molecules can keep the basic nitrogen functional groups protonated and this species would be responsible for the laser photon absorption. Therefore, it is important to know the photophysics of the selected compounds also in acidified solutions. In connection with this fact, for the piridoindole (β-carboline) MALDI matrix called harmine, the presence of the matrix as the protonated species in the solid sample was shown by fluorescence technique.[41]

As part of a comparative study of the photochemistry of the compounds described as matrices in UV-MALDI-MS, in this work we examined the photophysical processes occurring after the electronic excitation of these compounds in acetonitrile and acidified acetonitrile solutions, in air, oxygen and nitrogen atmospheres.

Results and discussion

Fig. 2 shows the UV-visible absorption spectra of HLA, AAN, 9AA and 3AQ. In general, the spectra obtained in acidified solutions are different from those obtained in acetonitrile solutions. As can be seen in Fig. 2, 3AQ and HLA showed the characteristic β-carboline bathochromic shift described elsewhere,[32–34] while AAN showed a hypsochromic shift. In the case of 9AA no effect was observed in acidified solutions because the 9AA hydrochloride hydrate was used.

The effect of acidification and oxygen content of the solution on the fluorescence emission and quantum yield was also studied (Table 1). As displayed in Fig. 2, HLA showed the same emission spectra in both solutions. 3AQ shows different emission spectra in acetonitrile and acidified acetonitrile solutions and both AAN and AAN + H^+ did not show any fluorescent emission.

Concerning the fact that the emission spectrum of HLA is the same in acetonitrile and acidified acetonitrile solutions, it is interesting to point out that, as is known, the acid–basic properties of β-carbolines, like HLA, were dramatically modified after photon absorption.[32] HLA showed high basicity in the fundamental state ($pK_a^{HLA} = 10.0$) and $\Delta pK = 13.0$ where $\Delta pK = pK^* - pK$, where pK was measured for the acid–base equilibrium in the ground state and pK^* for the same equilibrium in the electronic excited singlet state. The formation of the corresponding protonated species from HLA in the electronic excited singlet state has been previously described.[32–40]

By measuring the absorption spectra before and after laser shooting in the photoacoustic experiments, no photobleaching was observed for 3AQ, 9AA and HLA, in acetonitrile or acidified acetonitrile solutions, in air, oxygen or nitrogen atmospheres, showing good photostability for those compounds. In all the cases the photoacoustic signals as a function of time showed the same behaviour. No time shift, or shape changes were observed, with respect to the calorimetric reference. From this result it can be assumed that the lifetime of the triplet states of the studied compounds had values $\tau_T \geq \tau_R/5$ or $\tau_T \leq 5\ \tau_R$, where $\tau_R = 1$ μs is the resolution time of photoacoustic set up.[42,43]

In the case of AAN in acidified solution, an irreversible photobleaching of the irradiated samples was observed. The absorption spectra of the solutions showed a significant hypsochromic shift (λ_{max}, from 316 to 280 nm) and the amplitude of the measured acoustic signals changed, increasing the number of excitation pulses. Thus, photoacoustic determinations were not possible in this case.

The peak to peak amplitude of the first acoustic pulse (H) was used to measure the prompt heat released to the medium by the sample after excitation.

For the case of HLA, the behaviour of H as a function of the excitation fluence (F), measured at the same absorbance under three different atmospheres: air, nitrogen (N_2) and oxygen (O_2) showed a linear dependence, with the same slope. When compared with the calorimetric reference at the same sample absorbance, the same slope was obtained, as shown in Fig. 3. Good reproducibility of these measurements was obtained for different absorbances and at fluences $F < 25$ J m^{-2}. These results can be interpreted by using eqn (1):[44]

$$\frac{H}{F} = K\,\alpha\,(1 - 10^{-A}) \qquad (1)$$

where K is an experimental constant containing the thermoelastic properties of the solution and instrumental factors, A is the

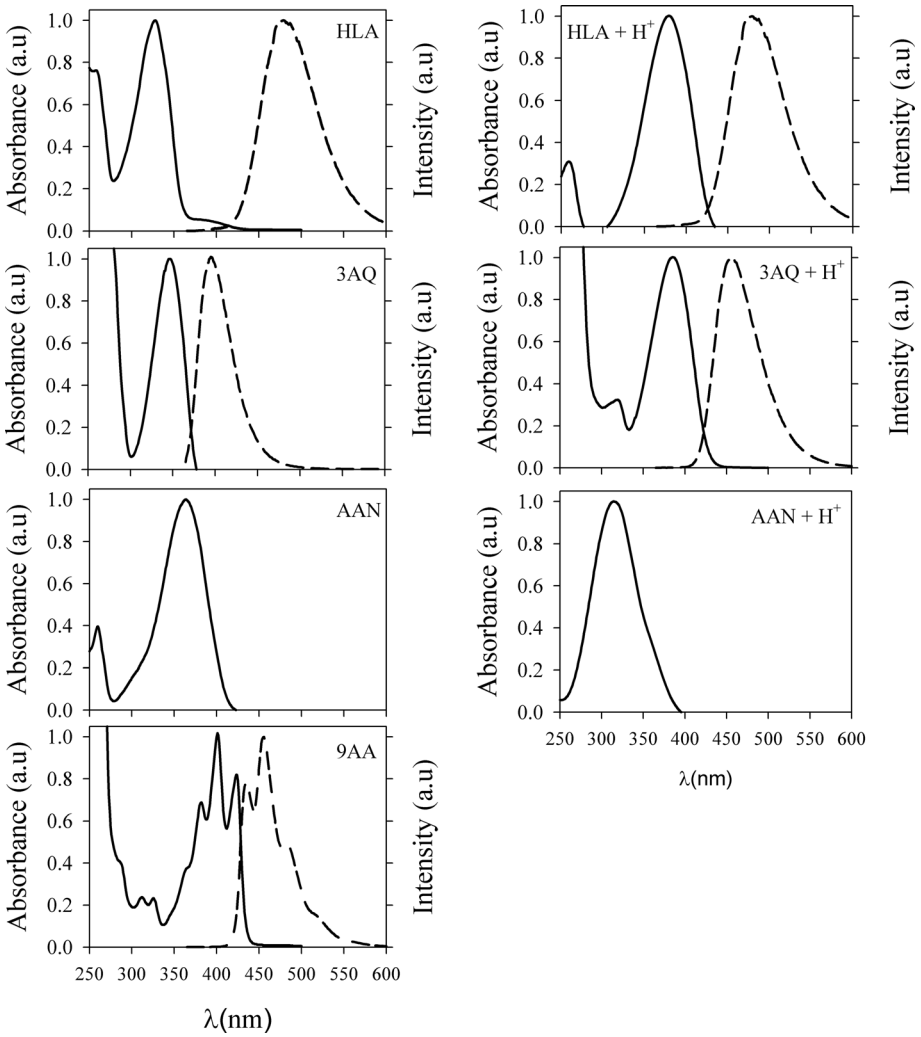

Fig. 2 UV-absorbance spectra (solid line) and fluorescence emission spectra (dashed line) of the compounds.

Table 1 Photophysical properties of the matrices studied

Matrix	Absorption data	Fluorescence data		Calorimetric data					Singlet oxygen data
	λ_a (nm)	λ_f (nm)	$\Phi_f O_2$ (±0.03)	$\Phi_f N_2$ (±0.03)	τ_T (μs) O_2	αO_2 (±0.02)	αN_2 (±0.02)	$\Phi_T N_2$	Φ_Δ (±0.05)
HLA	328	—	<0.03	<0.03	<0.2	1.00	1.00	nd[a]	<0.05
HLA + H^+	378	479	0.30	0.50	<0.2	0.78	0.63	nd[a]	<0.05
3AQ	347	393	0.10	0.16	<0.2	0.90	0.85	nd[a]	<0.05
3AQ + H^+	385	460	0.15	0.60	<0.2	0.88	0.42	>0.15	0.15
AAN	362	—	<0.03	<0.03	<0.2	0.99	0.86	>0.15	0.20
9AA + H^+	393, 403 and 426	429, 455, and 481 (sh)	0.61	0.84	<0.2	0.48	0.32	nd[a]	0.15

[a] nd: not determined.

sample absorbance value and α is the fraction of energy released to the medium as prompt heat, within the time resolution of the experiment.

Fig. 4 shows for HLA and calorimetric reference, the dependence of *H*/*F versus* the fraction of energy absorbed by the sample in an absorbance range of 0.100–0.230. As can be seen, linear plots were obtained. For HLA, slopes of sample and reference were the same. For HLA + H^+, in consistence with fluorescence data, slopes depended on the atmosphere and were different from the calorimetric reference. This special quenching

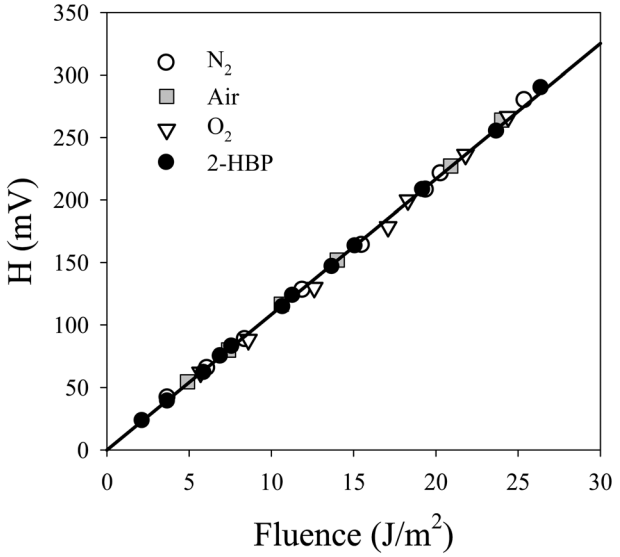

Fig. 3 Amplitude of the photoacoustic signals as a function of laser fluence for acetonitrile solutions of HLA (atmosphere: air, N_2, O_2) and 2-HBP (atmosphere: air).

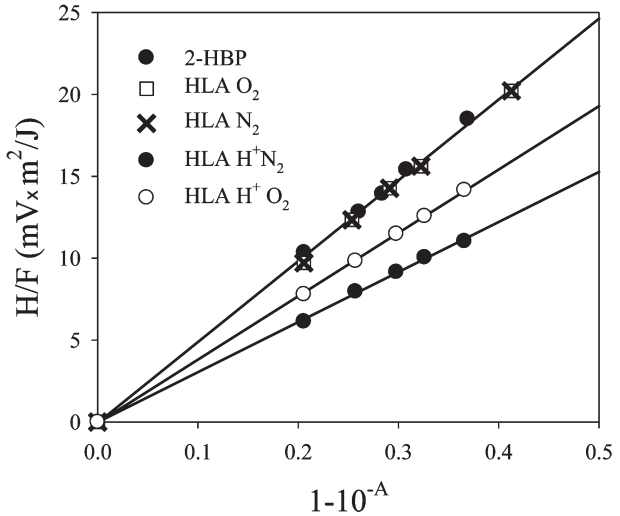

Fig. 4 Amplitude of the fluence-normalised photoacoustic signals as a function of the fraction of absorbed energy for HLA (neutral and acidified, atmosphere: N_2, O_2) and 2-HBP (atmosphere: air) in acetonitrile solutions.

of singlet excited states of some β-carbolines by O_2 was previously described.[22,32,45–47] From these plots, and taking into account that for the calorimetric reference 2-HBP $\alpha_R = 1$, the ratio of *H*/*F* values obtained for sample and reference yielded the sample α value. Thus, a value $\alpha = 1 \pm 0.02$, independent of the atmosphere, was obtained for HLA. This means that this molecule released to the medium all the absorbed energy as prompt heat—the heat integrated by the transducer—in processes faster than roughly $\tau_R/5$, as was previously discussed. We can assume that the lifetime of the triplet state of HLA is shorter than $\tau = 200$ ns. No evidence was found for the formation of photoproducts or intermediate species with lifetimes longer than this time (*i.e.* triplet state; phototautomers).

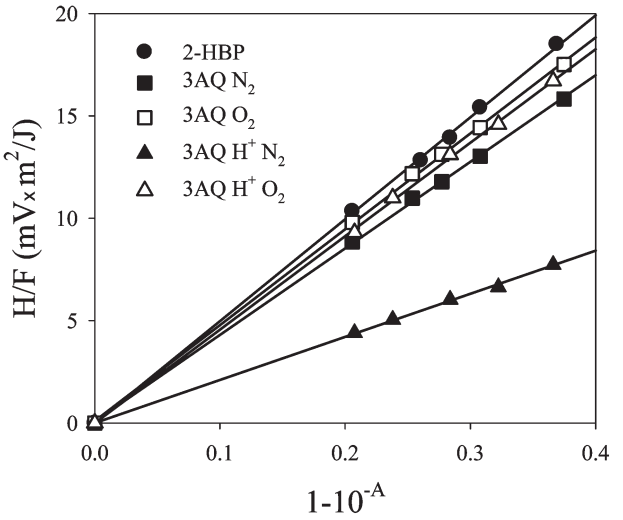

Fig. 5 Amplitude of the fluence-normalised photoacoustic signals as a function of the fraction of absorbed energy for 3AQ (neutral, atmosphere: N_2, O_2) and 2-HBP (atmosphere: air) in acetonitrile solutions.

The lack of oxygen effect for HLA in acetonitrile can be explained by taking into account the short lifetime of the involved singlet and triplet states. Then, any possible quenching effect would be of low efficiency and the sensitivity of the photoacoustic method may not be enough to detect any possible change.

Singlet oxygen phosphorescence measurements for this molecule in both acetonitrile and acidified acetonitrile solutions did not show any evidence of singlet oxygen formation in our experimental conditions.

For HLA + H^+, case values $\alpha_{O2} = 0.78 \pm 0.02$ and $\alpha_{N2} = 0.63 \pm 0.02$ were obtained. These values combined with fluorescence data fit the energy balance eqn (2):

$$E_a = \phi_f E_f + \alpha E_a + \phi_{ST} E_{ST} \quad (2)$$

where Φ_F is the fluorescence quantum yield, $\lambda_{exc} = hc/E_a$, is the excitation wavelength, $\lambda_f = hc/E_f$ is the fluorescence maximum wavelength measured experimentally (see Table 1), and the third term is the energy stored by species living longer than the heat-integration time, expressed as the product of quantum yield of formation, Φ_{ST} multiplied by the molar energy content E_{ST}. Then, as in the neutral form, independent of the atmosphere, we can assume that no energy storing species were present, and also in this case, the lifetime of the triplet state is shorter than 200 ns.

Fig. 5 shows for 3AQ and the calorimetric reference, the dependence of *H*/*F* *versus* the fraction of energy absorbed by the sample. Different slopes were obtained under different atmospheres (O_2, N_2) with values of $\alpha_{O2} = 0.90 \pm 0.02$ and $\alpha_{N2} = 0.85 \pm 0.02$ for the neutral form (3AQ) and $\alpha_{O2} = 0.88 \pm 0.02$ and $\alpha_{N2} = 0.42 \pm 0.02$ for the protonated form (3AQ + H^+).

In a nitrogen atmosphere, the third term of eqn (2) is the energy stored by the triplet state, which has a value: $\Phi_T E_T \cong 0$ in the neutral form and $\Phi_T E_T > 0$ in the protonated form of 3AQ. For the last one we can assume that the triplet state acted, in our photoacoustic experiments, as an energy storing species. By using simple energetic considerations to estimate upper and

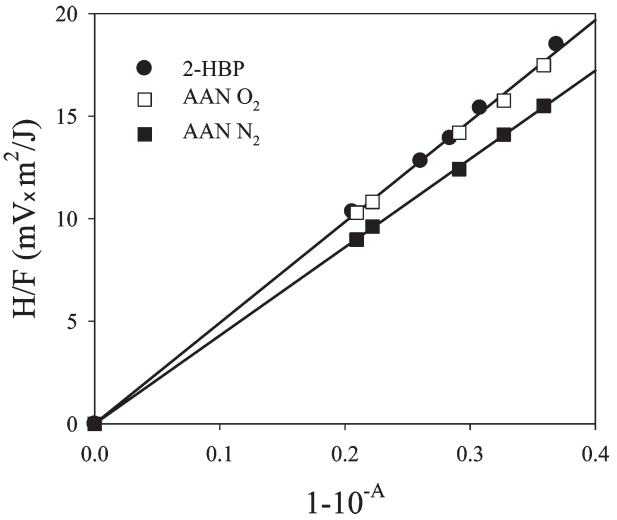

Fig. 6 Amplitude of the fluence-normalised photoacoustic signals as a function of the fraction of absorbed energy for neutral AAN (atmosphere: N_2, O_2) and 2-HBP (atmosphere: air) in acetonitrile solutions.

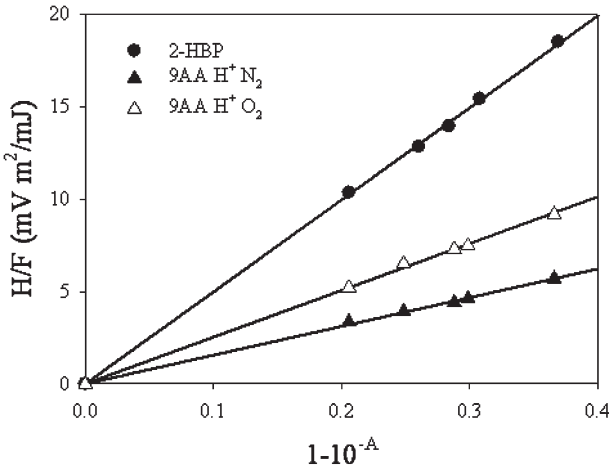

Fig. 7 Amplitude of the fluence-normalised photoacoustic signals as a function of the fraction of absorbed energy for 9AA hydrochloride (atmosphere: N_2, O_2) and 2-HBP (atmosphere: air) in acetonitrile solutions.

lower limits for E_T, $\Phi_T > 0.15$ can be estimated for 3AQ + H^+. In the case of the neutral form two possibilities can be considered. One is that the triplet state has a lifetime shorter than $\tau_R/5$ = 200 ns and all the energy storage in the triplet state is released to the medium as prompt heat. The other possibility is that the triplet state has a lifetime >5 μs and storage energy. In this situation, considering upper and lower limits for E_T, a value of $\Phi_T < 0.05$ can be estimated which is between the experimental uncertainty of the measurements.

Singlet oxygen formation was not detected in 3AQ, but it was observed in 3AQ + H^+ with an efficiency $\Phi_{\Delta 3AQ\ H+} = 0.15$ (Table 1). To determine if the triplet state is totally or partially quenched in an O_2 atmosphere, eqn (2) can be used again, assuming that now the energy storing species is singlet oxygen: ($\Phi_\Delta E_\Delta$, where E_Δ is known from the literature[43]). In these conditions, the equation corresponds to the case in which total quenching takes place. Then, by using the data obtained from the experiments performed in O_2 and singlet oxygen results, the energy balance can be checked and the quenching efficiency determined.

For both the neutral and protonated form of 3AQ, eqn (2) fits correctly within less than 5% of uncertainty. Then, it can be assumed that in an O_2 atmosphere, the triplet state of this compound is completely quenched in a time shorter than τ = 200 ns.

Fig. 6 shows the dependence of *H*/*F versus* the fraction of energy absorbed by the sample for AAN in the neutral form and the calorimetric reference. Also, in this case, different slopes were obtained under different atmospheres (O_2, N_2) with values of $\alpha_{O2} = 1.00 \pm 0.02$ and $\alpha_{N2} = 0.86 \pm 0.02$. By using eqn (2) and N_2 atmosphere data, $\Phi_T E_T > 0$ can be determined.

As in the case of HLA, the triplet state acted, in our photoacoustic experiments, as an energy storing species. Once upper and lower limits for E_T have been considered, $\Phi_T > 0.15$ can be estimated.

For this compound, singlet oxygen formation was observed with an efficiency $\Phi_\Delta = 0.20$ (Table 1). Then, by using eqn (2) and taking into account no fluorescence and the value of α determined, it can be concluded that in an O_2 atmosphere, the triplet state of this compound is completely quenched in a time shorter than 200 ns.

Fig. 7 shows the dependence of *H*/*F versus* the fraction of energy absorbed by the sample for 9AA hydrochloride and the calorimetric reference. $\alpha_{O2} = 0.48 \pm 0.02$ and $\alpha_{N2} = 0.32 \pm 0.02$ were determined. Singlet oxygen formation was observed for this compound with an efficiency $\Phi_\Delta = 0.15$ (Table 1). Eqn (2) also fits, in this case, with the corresponding data of Table 1, and we can conclude that in an O_2 atmosphere the triplet state of this compound is also completely quenched in a time shorter than 200 ns. By using eqn (2) and N_2 atmosphere data, $\Phi_T E_T \cong 0$ can be determined. Then, as in the case of 3AQ, we have again two possibilities: a triplet state with a lifetime shorter than 200 ns or with a lifetime >5 μs and $\Phi_T < 0.05$.

Experimental

Spectrograde and HPLC grade acetonitrile was purchased from J. T. Baker and was used without further purification. 2-Hydroxybenzophenone (2-HBP), phenalenone (PH), 9-aminoacridine hydrochloride hydrate (9AA), 3-aminoquinoline (3AQ), 2-(2-aminoethylamino)-5-nitropyridine (AAN) and 3,4-dihydro-7-methoxy-1-methyl-9*H*-pyrido[3,4-*b*]indole (harmaline, HLA) were purchased from Sigma Aldrich and were used without further purification.

Experiments were performed in acetonitrile and acidified acetonitrile solutions (with H_2SO_4). Since the compounds under study have different pK_a values, the H_2SO_4 amount added to acetonitrile solutions was regulated in order to obtain the protonated form of the studied compounds.

The UV-visible absorption measurements were recorded with a UV-visible Shimadzu UV-1203 spectrophotometer. All the measurements were made with 1 cm stopped quartz cells at 298 K. Steady-state fluorescence measurements were performed at 298 K using a spectrofluorimeter Cary Eclipse (Varian). The fluorescence quantum yields were determined from the corrected

fluorescence spectra using quinine bisulfate (Purest) in 0.5 M H_2SO_4 as reference[48] (Φ_F = 0.546).[49] To avoid inner filter effects, the absorbance of the solutions, at the excitation wavelength (355 nm), was kept below 0.10.

Photoacoustic measurements were performed by using a set-up already described.[50] A Q-Switched Nd:YAG laser (7 ns FWHM) operating at 355 nm was used as excitation source (1 mm diameter in the cell). A home-made ceramic PZT (4 × 4 mm) transducer with an appropriate amplifier was used to detect the acoustic signals. The resolution time of the experiments, determined by our experimental set-up, was τ_R = 1 μs.

Measurements were performed averaging the acoustic signals generated by 64 laser shots for better signal to noise ratio. The UV-vis spectrum of the solutions was checked before and after each set of laser shots. 2-HBP was used as a calorimetric reference (CR).[51] For the experiments, sample and reference solution concentrations were matched to absorbance values between 0.1 and 0.2 at the laser wavelength. Experiments were performed at open air and under a controlled atmosphere, bubbling N_2 or O_2 in the solution, for 15 min.

Time resolved phosphorescence detection was used for singlet oxygen detection. The near IR luminescence of O_2 ($^1\Delta_g$) was observed at 90° geometry through a 5 mm thick AR coated silicon metal filter with a wavelength pass >1.1 μm and an interference filter at 1.27 μm by means of a preamplified (low-impedance) Ge-photodiode (Applied Detector Corporation, time resolution 1 μs). Simple exponential analysis of the emission decay was performed with the exclusion of the initial part of the signal. Phenalenone, with $\Phi_{\Delta Ph}$ = 0.95[52] was used as reference.

Conclusions

The results obtained in this work show that all the samples in their neutral form and 9AA hydrochloride (9AA + H^+) are highly efficient systems for the release of the absorbed energy as heat by non radiative processes. In acid solutions, HLA and 3AQ show important changes in their photophysical behaviour with respect to neutral solutions, increasing their fluorescence and diminishing with respect to calorimetric efficiency.

For all the studied compounds, both in the neutral or protonated form, the triplet state was quenched in oxygen atmosphere or air in a time shorter than 200 ns. In these atmospheres, the compounds had their highest prompt heat efficiency. This means that they are potentially good candidates to improve MALDI sources at atmospheric pressure (AP) MALDI.

Singlet oxygen formation was not detected for HLA in both acetonitrile and acidified acetonitrile solutions and 3AQ in acetonitrile. On the contrary, both AAN and 9AA in acetonitrile and 3AQ in acidified acetonitrile solution showed clear evidence of singlet oxygen production with efficiencies between 0.15 and 0.20.

In a nitrogen atmosphere, triplet states lifetimes seemed to be enlarged in 3AQ + H^+ and AAN. In these cases the triplet state acts as an energy storing species also diminishing the calorimetric efficiency.

MALDI ionization is a complex phenomenon because of the strong interaction between the physical and chemical components of the process. Desorption and ablation by phase explosion may all be active in a MALDI experiment, but it is not clear which are the species responsible for these events. Although our results were obtained in solution, if the studied compounds keep a similar behaviour in the solid state, they should be good candidates for induced thermal ablation from electronic excited states. Under this assumption HLA, AAN, 3AQ are potentially better matrices than 9AA. However, in UV-MALDI MS experiment (results not shown) we observed that AAN is not a very efficient matrix, probably because of its photodegradation during analysis.

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