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Triplet state energy of the carotenoid bixin determined by photoacoustic calorimetry

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Dedicated to Professor Beatriz Lopez de Mishima (Universidad Nacional de Santiago del Estero, Argentina) on the occasion of her 60th birthday.

Abstract

The triplet state energy level of the natural *cis*-carotenoid bixin (methyl hydrogen 9′*Z*-6,6′-diapocarotene-6,6′-dioate) was determined using laser-induced photoacoustic calorimetry (PAC) in acetonitrile/methanol (1:1) solution. An energy-transfer process with anthracene as sensitizer in O_2-saturated solution was used to populate the carotenoid triplet state. The energy balance of the system allowed the calculation of the triplet energy content of bixin as $E_T = 18 \pm 2$ kcal/mol. The result is discussed in relation with both its singlet molecular oxygen quenching and *cis* → *trans* isomerization abilities. The quantum yield, $\Phi = 0.69$, for the formation of either the triplet state or the singlet molecular oxygen by the sensitizer anthracene was also determined.

Keywords: Bixin; Carotenoids; Triplet state; Energy-transfer; Photoacoustic calorimetry (PAC)

1. Introduction

Bixin (methyl hydrogen 9′*Z*-6,6′-diapocarotene-6,6′-dioate), Fig. 1, is the main carotenoid found in the seed coat of the fruits from annatto tree (*Bixa orellana* L.), which is cultivated in tropical regions of South and Central America, Africa and Asia [1,2]. Annatto extracts are widely used in the food industry as natural coloring agents in cheese, ice cream, yoghurt, sausages, margarine, snacks and dressings, and compared to other natural colors are relatively inexpensive, thus increasing their commercial relevance [1,2].

Bixin is one of the few naturally occurring *cis*-carotenoids, whose formation is thought to occur by the oxidative degradation of the parent C_{40}-carotenoid [3–5]. Almost all carotenoids are found in Nature, bixin is an efficient quencher of singlet molecular oxygen $O_2(^1\Delta_g)$ in fluid solutions, with bimolecular quenching rate constant similar to that observed for β-carotene [6–9]. Since the quenching of $O_2(^1\Delta_g)$ by carotenoids is due to an efficient energy-transfer process [10], the above result indicates that the triplet energy level of bixin should be lower than that of the energy level of $O_2(^1\Delta_g)$, e.g. 22.5 kcal/mol [11].

In addition, the thermo-stability of the 9′-*cis* double bond of bixin was reported as being so great that the molecule underwent a second *trans* → *cis* isomerization at the C-9, giving considerable amounts of 9,9′-di-*cis* isomer [12–14]. In fact, the activation energy E_a for the thermal 9-*trans* → *cis*

Abbreviations: ANT, Anthracene; HBP, 2-hydroxibenzophenone; $O_2(^3\Sigma_g^-)$, ground state molecular oxygen; $O_2(^1\Delta_g)$, singlet molecular oxygen; ^{3}Bix*, bixin triplet excited state; HLPC, high-performance liquid chromatography; PDA, photodiode array detection; LFP, laser-flash photolysis; PAC, laser-induced photoacoustic.

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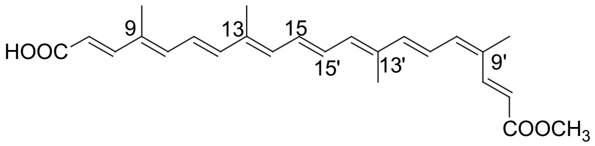

Fig. 1. Chemical structure of the natural *cis*-carotenoid bixin.

was calculated as 15 kcal/mol, as compared to 24 kcal/mol for the thermal 9′-*cis* → *trans* isomerization in aqueous media [15], confirming with the quantitative thermodynamic data of the previous observations.

On the contrary, in photosensitized reactions using Rose Bengal or Methylene Blue as sensitizers only quantitative 9′-*cis* → *trans* isomerization was observed, with a much lower $E_a = 6$ kcal/mol [9]. In this case, the quantitative formation of *all-trans* bixin was mediated by the excited triplet state of bixin, ^{3}Bix*, either under anaerobic or aerobic conditions [9].

Since many of the biological and functional properties of the carotenoids are related with their triplet excited states [10], in the present work the triplet energy level of bixin was calculated using laser-induced photoacoustic calorimetry (PAC), which is a suitable technique for the calculation and characterization of energy levels of photoinduced excited states [16–18].

2. Experimental

2.1. Materials

Bixin was extracted with ethyl acetate from annatto seeds previously washed with hexane and methanol. The solvent was completely evaporated on a rotary evaporator, followed by crystallization from dichloromethane/ethanol (1:4), repeated twice. The annatto crystals obtained showed 97% purity, indicated by high-performance liquid chromatography-photodiode array detector (HPLC-PDA). Anthracene (ANT) and 2-hydroxybenzophenone (HBP) were from Aldrich (Milwaukee, USA) and were recrystallized from ethanol before use. All the organic solvents used were of HPLC grade from Sintorgan (Buenos Aires, Argentina). N_2 and O_2 were of high purity (99.99%) from Indura (Córdoba, Argentina).

2.2. Methods

Stationary UV–vis spectra were obtained with a Hewlett-Packard 8453 diode array spectrophotometer. Fluorescence spectra were recorded with a Hitachi F-2500 instrument. Fluorescence quantum yields of the sensitizer anthracene under N_2- and O_2-saturated solutions were determined using quinine sulfate in 1 N H_2SO_4 as actinometer ($\Phi_F = 0.57$), and correcting by refractive index of the solvents [19].

HPLC analysis was carried out using a Waters HPLC system equipped with a photodiode array detector (Waters, model 996) as described elsewhere [9,15].

Laser-induced photoacoustic calorimetry (PAC) experiments were performed using a Continuum Minilite II Nd–YAG laser generating 355 nm pulses (FWHM 10 ns) as excitation source of the sensitizer anthracene. The laser beam width was shaped with a rectangular slit (0.5 mm width × 5 mm high), which allows a transit time of ca. 0.4 μs for the acoustic wave in the organic mixture. Under this condition, all transient species with lifetimes longer than 2 μs can be considered as final products for the photoacoustic experiment [16]. The maximum total laser energy after the slit was <120 μJ/pulse, measured with a pyroelectric energy meter Melles-Griot model 13PEM001. An aqueous solution of $Na_2Cr_2O_7$ of different transmittance (5–90% T) was used as filter in order to abate the laser energy and it performs energy-dependence measurements. The pressure wave was detected with a ceramic piezoelectric transducer spring-loaded to the cuvette window parallel to the laser beam direction. The signals were amplified 10 times (Comlinear E103) and fed into a transient recorder Tektronik TDS 3032B. In order to avoid sensitizer consumption, no more than 10 single signals were averaged. Deconvolution analysis of the signals was performed with the Sound Analysis 3000 v.1.13 software (Quantum Northwest Inc., Spokane, WA, USA) according to procedures published elsewhere [16,17,20]. In all cases, the absorbances of ANT and of the calorimetric reference HBP at 355 nm were matched within ±2%. The concentration of bixin was lower than 9 μM in order to avoid the absorption of the laser light by the carotenoid.

Transient absorption spectrum of the triplet state of bixin in O_2-saturated solutions was recorded with a Luzchem m-LFP 112 system, using the same laser source that was used for the PAC experiments. Since in this case the incident laser power was much higher (ca. 5 mJ/pulse), single shot transient decays were recorded at each wavelength in order to minimize the sample degradation. An average transient spectrum was obtained after four independent sets of experiments.

The experiments under anaerobic or aerobic conditions were performed by bubbling solvent-saturated N_2 or O_2, respectively, for 20 min. The temperature was set at 25.0 ± 0.1 °C.

3. Results and discussion

Fig. 2 shows the photoacoustic signals for the calorimetric reference HBP and for the sensitizer ANT observed after 355 nm laser excitation under identical experimental conditions in N_2- and O_2-saturated ACN:MeOH (1:1) solutions. No significant delay of the sample signal relative to the reference was observed, indicating that the PZT detector is sensing only prompt pressure changes. Deconvolution analysis of the signals confirmed the presence of a main prompt component (<10 ns, data not shown). In these cases, the amplitude signal of the first wave is quantitatively related with the prompt formation of transient species with lifetimes much longer than that of the upper time resolution of the PZT detector.

Fig. 3 shows the plots of the amplitude of the first wave of the photoacoustic signal, H, vs. the absorbed laser energy, E_a. In all cases, the plots were linear with zero intercepts, indicating that either bi-photonic or signal saturation processes do not take place. Table 1 lists the observed slopes for the linear plots of Fig. 3, together with the reference-to-sample ratio values.

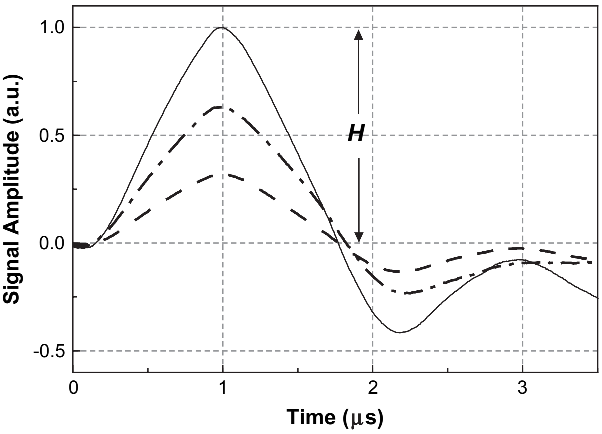

Fig. 2. Reference-normalized optoacoustic signals of the calorimetric reference 2-hydroxibenzophenone (solid line), and of anthracene in N_2- (dashed line) and in O_2-saturated (dash-dot line) ACN:MeOH (1:1) solutions.

In photoacoustic techniques, the amplitude of acoustic signal of the sample, H_S, contains information of both the thermal ΔV_{th}, and structural ΔV_{str} volume changes produced after the absorption of light [16]. The ΔV_{th} contribution is related to the solvent expansion due to the heat released in the non-radiative decay processes of the excited states and only depends on the thermoelastic parameters of the solvent. In turn, the ΔV_{str} is due to the contraction or expansion produced by molecular rearrangements (solute and solvent) in the photochemical reaction. Thus, the H_S is expressed by Eq. (1):

$$H_S = K(\alpha \Delta V_{th} + \Delta V_{str}) = K\left(\alpha E_a \frac{\beta}{c_p \rho} + \Delta V_{str}\right) \tag{1}$$

where K is a proportionality constant that includes geometric and electronic parameters of the detection system, α is the released heat fraction of the absorbed energy. β, c_p and ρ are the thermoelastic parameters of the medium, i.e. the isobaric thermal expansion coefficient, the specific heat capacity and the density, respectively. The ratio $(\beta/c_p\rho)$ is often called the adiabatic expansion coefficient.

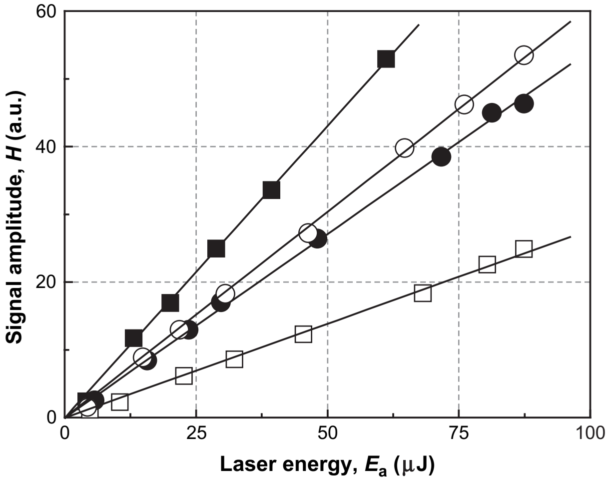

Fig. 3. Maximum signal amplitude (H) vs. the absorbed laser energy (E_a): (■) 2-hydroxibenzophenone; (□) anthracene, N_2-saturated; (●) anthracene, O_2-saturated; (○) anthracene, O_2-saturated + 5 μM bixin.

Table 1
PAC parameters obtained at 25 °C in ACN:MeOH (1:1) solutions

Solution	Slope (a.u.)/10^4	r	$\alpha = H_S/H_R$
HBP (reference)	8.61 ± 0.07	0.99959	1.000 ± 0.016
Anthracene + N_2	2.77 ± 0.04	0.99942	0.322 ± 0.007
Anthracene + O_2	5.42 ± 0.05	0.99916	0.630 ± 0.011
Anthracene + O_2 + 5 μM bixin	6.07 ± 0.04	0.99976	0.706 ± 0.010
Anthracene + O_2 + 9 μM bixin	6.34 ± 0.05	0.99956	0.736 ± 0.012

On the other hand, it is assumed that the calorimetric reference compound in the same medium releases all the absorbed energy as prompt heat ($\alpha = 1$) and with zero structural volume changes, e.g. $\Delta V_{str} = 0$. Thus, the signal amplitude for the calorimetric reference H_R only depends on the absorbed energy E_a and the adiabatic expansion coefficient, $(\beta/c_p\rho)$. For the reference and sample solutions with absorbance values matched at the excitation wavelength, the ratio between the sample and reference amplitude signals yields the following relationship, Eq. (2):

$$\frac{H_S}{H_R} = \alpha + \frac{\Delta V_{str}}{E_a}\left(\frac{c_p \rho}{\beta}\right) \tag{2}$$

In organic solvents at room temperature, the $(c_p\rho/\beta)$ ratio is relatively small (<0.5 kcal/cm^3). In addition, if the photochemical process produces minimal structural changes, e.g. $\Delta V_{str} \approx 0$, the second term at the right side of Eq. (2) is negligible and $H_S/H_R \approx \alpha$. In this case, the photoacoustic technique behaves as a fully photocalorimetric method [16,21]. The α value provides a valuable insight into the photophysical properties of the sample molecule. Thus, the absorbed energy per laser photon E_λ ($\equiv$80.5 kcal/mol at 355 nm) provides the sum of the energy released as prompt heat, plus the energy lost in fast radiative processes, e.g. fluorescence, and the energy 'stored' in a long-lived transient such as a triplet state, Eq. (3):

$$E_\lambda = \alpha E_\lambda + E_F \Phi_F + E_{st} \Phi_{st} \tag{3}$$

where $E_F\Phi_F$ is the average energy released as fluorescence and $E_{st}\Phi_{st}$ is the energy stored by a long-lived transient.

In order to prove the above assumptions and the experimental PAC set-up, the photophysical formation of the triplet state of anthracene (ANT) under anaerobic conditions was evaluated, Eq. (4):

$$\mathrm{ANT} \xrightarrow{h\nu} {}^1\mathrm{ANT}^* \xrightarrow{\Phi_T} {}^3\mathrm{ANT}^* \tag{4}$$

In N_2-saturated solution the long-lived ^{3}ANT* (>100 μs, [11]) exceeds by two orders of magnitude the transit time (≈0.4 μs) of the PAC set-up. Therefore, the ^{3}ANT* is by far the energy-storing species, and its triplet quantum yield Φ_T was calculated as:

$$\Phi_T = \frac{E_\lambda(1 - \alpha_{N_2}) - E_F \Phi_{F,N_2}}{E_T} = 0.69 \tag{5}$$

with $E_F = 76$ kcal/mol and $E_T = 42.5$ kcal/mol [11] and $\Phi_{F,N_2} = 0.33$ as determined by fluorescence actinometry (see Section 2, data not shown). The obtained $\Phi_T = 0.69$ for ^{3}ANT* is coincident with the literature data [11,22], confirming all the above assumptions and indicating that the structural volume changes associated with the triplet state formation of anthracene in organic media are negligible.

On the other hand, in O_2-saturated organic solutions, the oxygen concentration is ca. 10 mM, and under this condition the long-lived ^{3}ANT* is efficiently quenched by molecular oxygen, $k_q = 4 \times 10^9\ M^{-1}\ s^{-1}$ [11], by an energy-transfer process producing singlet molecular oxygen $O_2(^1\Delta_g)$, Eq. (6).

$$^3\text{ANT}^* + O_2\left(^3\Sigma_g^-\right) \xrightarrow{\Phi_\Delta} \text{ANT} + O_2\left(^1\Delta_g\right) \tag{6}$$

In ACN:MeOH (1:1) solutions, the $O_2(^1\Delta_g)$ lifetime was reported to be about 15 μs [9], exceeding the upper time resolution of the PAC experiment (2 μs). Therefore the experimental PAC slope ratio, α_{O_2}, represents the fraction of prompt heat released on the formation of $O_2(^1\Delta_g)$, allowing the calculation of $O_2(^1\Delta_g)$ quantum yield with anthracene as photosensitizer, Φ_Δ, Eq. (7):

$$\Phi_\Delta = \frac{E_\lambda(1 - \alpha_{O_2}) - E_F \Phi_{F,O_2}}{E_\Delta} = 0.68 \tag{7}$$

where $\Phi_{F,O_2} = 0.19$ as determined by fluorescence actinometry and $E_\Delta = 22.5$ kcal/mol [11]. It is observed that $\Phi_T = \Phi_\Delta \approx 0.7$, indicating that 100% of the anthracene triplet states quenched by molecular oxygen produces $O_2(^1\Delta_g)$, e.g. quenching efficiency of 1. This result also confirms that the generation of $O_2(^1\Delta_g)$ by energy-transfer quenching of triplet states does not produce important structural volume changes. In fact, Feitelson and Mauzerall have reported that the formation of $O_2(^1\Delta_g)$ was accompanied by a small contraction of $-0.2\ cm^3$/mol in the energy-transfer quenching of porphyrin derivatives in aqueous media [23], a very close value to the typical experimental error bars for ΔV determinations in photoacoustic experiments [16,17,20].

In the presence of bixin, $O_2(^1\Delta_g)$ is efficiently quenched by an energy-transfer process with diffusional controlled rate constant, $k_{q,\Delta} = 1.3 \times 10^{10}\ M^{-1}\ s^{-1}$ [9], Eq. (8):

$$O_2(^1\Delta_g) + \text{Bix} \xrightarrow{\Phi_T^{\text{Bix}}} O_2\left(^3\Sigma_g^-\right) + {}^3\text{Bix}^* \tag{8}$$

The photosensitized formation of ^{3}Bix* in O_2-saturated solutions was tested by transient absorption spectroscopy, Fig. 4. The transient spectra observed at 4 μs after the laser pulse showed the typical absorption band at 520 nm assigned to the triplet state of the carotenoid [24]. In this case, the quantum yield for the formation of ^{3}Bix*, Φ_T^{Bix} is given by:

$$\Phi_T^{\text{Bix}} = \eta_{q,\Delta} \times \Phi_\Delta = \frac{k_{q,\Delta}\tau_\Delta^0[\text{Bix}]}{1 + k_{q,\Delta}\tau_\Delta^0[\text{Bix}]} \times \Phi_\Delta \tag{9}$$

where $\eta_{q,\Delta}$ is the quenching efficiency of $O_2(^1\Delta_g)$ by bixin. In turn, the quenching efficiency depends on the $O_2(^1\Delta_g)$ lifetime τ_Δ, the quenching rate constant $k_{q,\Delta}$, and the carotenoid concentration. Therefore, using $\tau_\Delta^0 = 15$ μs and $k_{q,\Delta} = 1.3 \times 10^{10}\ M^{-1}\ s^{-1}$ [9], $\Phi_T^{\text{Bix}} = 0.34$ and 0.45 were obtained at [Bix] = 5 μM and 9 μM, respectively.

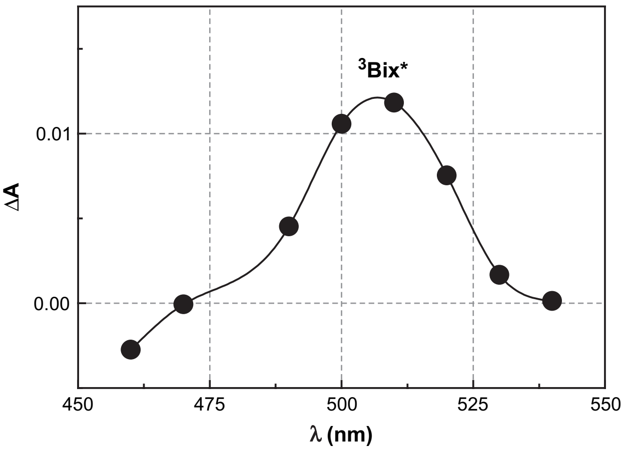

Fig. 4. Transient absorption spectra of the triplet state of bixin, obtained after 4 μs of the 355 nm laser excitation of anthracene in the presence of 9 μM bixin in O_2-saturated ACN:MeOH (1:1) solution.

In the presence of bixin in O_2-saturated solutions, the PAC slope ratios $\alpha_{O_2}^{\text{Bix}}$ were somewhat larger than in the absence of carotenoid, Table 1. For both $O_2(^1\Delta_g)$ and ^{3}Bix* the loss of energy by phosphorescence can be neglected, since for most organic molecules the phosphorescence quantum yield is very low ($\Phi_P < 10^{-3}$) in fluid solution and at room temperature. Therefore, the increment of PAC slope by the addition of carotenoid in the anthracene O_2-saturated solutions is due to energy-transfer Eq. (8), resulting in the formation of ^{3}Bix* from $O_2(^1\Delta_g)$. In this case, the PAC slope difference is proportional to the energy released in the ^{3}Bix* formation. e.g. $E_T^{\text{Bix}}\Phi_T^{\text{Bix}}$, Eq. (10):

$$E_\lambda\left(\alpha_{O_2}^{\text{Bix}} - \alpha_{O_2}\right) = E_T^{\text{Bix}}\Phi_T^{\text{Bix}} \tag{10}$$

The last equation allowed the calculation of $E_T^{\text{Bix}} = 17.6 \pm 1.8$ kcal/mol and 18.9 ± 2.0 kcal/mol at 5 μM and 9 μM of bixin, respectively, and an average value of $E_T^{\text{Bix}} = 18 \pm 2$ kcal/mol can be considered. This result is similar to the triplet energy of β-carotene of 19.5 kcal/mol, also obtained with PAC [18]. For both carotenoids, the E_T level is lower than that of the energy content of $O_2(^1\Delta_g)$ (22.5 kcal/mol) allowing down-hill energy-transfer process with diffusional quenching rate constants, as observed for both carotenoids in reversed micelles solutions, e.g. $k_q^\Delta \approx 1.3 \times 10^{10}\ M^{-1}\ s^{-1}$ [8].

In a previous work, we have reported that the photosensitized 9′-*cis* → *trans* isomerization of bixin in ACN:MeOH mixtures was mediated by ^{3}Bix*, with $E_a = 6$ kcal/mol [9]. This means that the crossing point with the potential surface of the *all-trans* isomer is ca. 24 kcal/mol from the bixin ground state. Recently, the kinetics and mechanism of the thermal degradation of bixin was studied in ethanol/water mixtures, allowing the determination of 24 kcal/mol for the activation energy of the thermal 9′-*cis* → *trans* isomerization [15]. In spite of the different solvents, the triplet energy should remain almost constant and therefore $E_{a,th} \approx E_T^{\text{Bix}} + E_{a,ph}$, as it is depicted in Fig. 5.

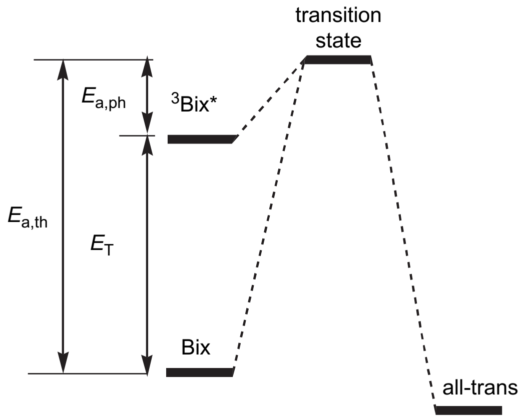

Fig. 5. Energy-level scheme associated with the triplet state mediated photosensitized ($E_{a,ph} = 6$ kcal/mol) and thermal ($E_{a,th} = 24$ kcal/mol) *cis* → *trans* isomerization of bixin in polar solvents.

In summary, laser-induced photoacoustic calorimetry (PAC) has been showed as a suitable technique for the determination of triplet energies of carotenoids populated by photosensitized energy-transfer processes in O_2-saturated solutions. The $E_T^{Bix} = 18 \pm 2$ kcal/mol accommodates well with other features of the triplet state photochemistry of bixin, such as its $O_2(^1\Delta_g)$ quenching ability. In addition, no important structural volume changes ($\Delta V_{str} \approx 0$) were observed for any of the transient species formed during the energy-transfer processes. This fact can be useful in order to use the laser-induced photoacoustic technique as a calorimetric method in energy-transfer processes avoiding extra protocols or corrections for the separation of the structural volume contribution to the acoustic signal.

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