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ORIGINAL PAPER

Spectroscopic Properties and Laser Induced Fluorescence Determination of Some Endocrine Disrupting Compounds

Badr Benmansour · Ludovic Stephan · Jean-Yves Cabon · Laure Deschamps · Philippe Giamarchi

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Abstract This work presents spectroscopic properties of some Endocrine Disrupting Compounds (EDCs), frequently found in food and in natural water. Studied molecules belong to the groups of phenolic and phthalate EDCs. In a first part, we have examined their absorption and fluorescence properties. Fluorescence emission wavelengths are about 300 nm for phenolic compounds and 360 nm for phtalate compounds; main excitation wavelengths being comprised between 210 nm and 230 nm. Fluorescence lifetimes measured are short (about 4 ns) and the fluorescence quantum yield has been determined. In a second part, to avoid the time consuming solvent extraction step, an analytical application to evaluate the performance of a direct analysis by laser induced fluorescence spectroscopy of ECDs traces in tap water and in raw water is presented. Good detection limits have been obtained, i.e.: 0.35 $\mu g.L^{-1}$ of chlorophenol in tap water, which are always lower than the reported Predictive Non Efficient Concentration (PNEC).

Keywords Fluorescence · Laser · Spectroscopy · Phtalate · Phenolic compounds · Endocrine disrupting compounds

Introduction

Endocrine Disrupting Compounds (EDCs) are molecules having hormono-mimetic properties that disrupt hormonal balance and lead to adverse health effects including the alteration of reproductive functions [1]. Around years 1950, observations were made for animals, such as the reduction of the reproduction rate of various avian species (sterns of the Dutch littoral or pelicans born in the United States), or the masculinisation of the females of marine gastropods. They were, for the first time, attributed to some hormono-mimetic substances polluting the ecosystems (organochlorinated pesticides, antifouling paintings containing tributyltin). In mankind, an event consecutive to the exposure of pregnant women to distilbene (a synthetic oestrogen employed to prevent miscarriages) has induced disorders in fertility. In the seventies, the exposure of workers manufacturing chlordecone (an insecticide with oestrogenic properties) generated neurologic and fertility disorders. The denomination "Endocrine Disrupters" appeared in 1991 [2]. It indicates any chemical substance, of natural or artificial origin, which can interfere with synthesis, storage, transport, action or elimination of natural hormones. These molecules act at very low doses, consequently, they do not induce a direct toxic effect, but rather a discrete disturbance which can be difficult to highlight [3–5].

The EDCs can be classified in two main classes of compounds i.e.: natural and synthetic.

Among the natural compounds, we found lignanes (from vegetal) and phyto-oestrogens or isoflavonoids (from soya). They have an oestrogenic activity, disturbing the development of female sexual characters.

The synthetic substances are used in industry (detergents, rust preventives, plasticizers,…), in agriculture (pesticides, herbicides, insecticides, fungicides), and in life products (cosmetics, cleaning products, food packaging).

Our work focused on two groups of synthetics compounds among these molecules: phenolic compounds and phthalates. These compounds may be contaminant in food,

B. Benmansour · L. Stephan · J.-Y. Cabon · L. Deschamps · P. Giamarchi (✉)
Brest University, UMR CNRS 6521 CEMCA,
Université Européenne de Bretagne,
6 av. Le Gorgeu,
29285 Brest cedex 3, France
e-mail: philippe.giamarchi@univ-brest.fr

through the plastics packaging, plastic water bottles and baby's bottles [6]. They are also found in large amounts in environment by the decomposition of detergents [7]. They are persistent molecules and they have a lipophilic character, so they can be stored in animal lipidic tissue and are difficult to eliminate [8].

Phenolic compounds are generally present in wastewater. We find mainly:

- Alkylphenols, coming from the alteration of alkylphenol polyetoxylates (detergents). The four most abundant in wastewater and in environment are: 4-n-heptylphénol; 4-n-nonylphénol; 4-tert-butylphénol; 4-tert-octylphénol [9].
- Bisphenol A, which is widely used in the production of polycarbonate bottles (for baby feeding) and kitchenware resisting to boiling temperature. It is also used in epoxy resins for internal surface of beverage and food cans [10, 11].
- Chlorophenol frequently found in wastewaters [12].

Phthalate compounds are commonly used as plasticizers in plastic production (particularly in PVC) to make them flexible. The main compounds used are [13]:

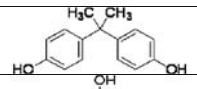
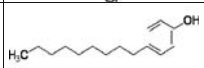
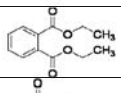
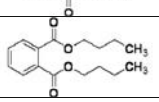
- Terephtalic Acid, used for the plastic bottles in polyethylene terephthalate;
- Di (2-ethylhexyl) phtalate, di isononylphtalate, and di nButyl Phtalate, used as a plasticizer.

Among the most encountered EDCs, we have selected seven compounds for this study (phenol, bisphenol A, chlorophenol, nonylphenol, diethyl phtalate (DEP), dibutyl phtalate (DBP) and phtalic acid); their formula and main physicochemical properties are detailed in Table 1 (except for phtalic acid).

From the literature it appeared to us that the spectroscopic properties of these ECDs compounds are not well known. Consequently, in a first part, we chose to study their absorption and fluorescence properties. First, we determined the absorbance maxima and the molar extinction coefficients. Then, we recorded the fluorescence excitation/emission matrix of each molecule in order to determine their characteristic excitation/emission couples. Using time resolved laser induced fluorescence, we measured the fluorescence lifetime of these compounds. Then, we determined their fluorescence quantum yield by reference to phenol.

In a second part, an analytical application has been examined to evaluate a direct analysis by laser induced fluorescence spectroscopy of ECDs traces in tap water and in raw water. As drinking water and environmental controls are an important problem, classical analytical methods have been developed for ECDs determination [14]. For their analysis, a solvent extraction step followed by a chromatographic separation and a mass spectrometry detection has been used. These protocols are time consuming and present some risk of sample contamination by storage flask. To determine these compounds by direct analysis, we used a pulsed YAG laser with an Optical Parametric Oscillator as a high energy excitation source and an ICCD camera for highly sensitive fluorescence detection [15, 16]. The results obtained and the performance of the laser induced fluorescence method are discussed.

Table 1 Endocrine disrupting compounds studded: spectroscopic properties and some physicochemical characteristics [28, 29]

Compound	Formula	Log Kow [28, 29]	PNEC ($\mu g.L^{-1}$) [10]	Excitation / Emission (nm/nm)	Molar extinction ($L.cm^{-1}.mol^{-1}$) λ_{nm} → ε	Detection Limit ($\mu g.L^{-1}$) (λ_{EX}=230nm) Tap water	Detection Limit ($\mu g.L^{-1}$) (λ_{EX}=230nm) Sea water	Fluo. lifetime (ns)	Fluo. quantum yield Φ (λ_{Ex}=225nm)
Phénol		1.46		212 / 300 270 / 300	212 → 6 439 225 → 2 411 270 → 1 729	0.45	4.2	3.85	0.22
Bisphénol A		3.32	64	226 / 305 276 / 305	225 → 13 764 276 → 3 213	2.0	23	3.27	0.010
Chlorophenol		2.39		225 / 310 279 / 310	225 → 13 454 279 → 2 579	0.35	8	4.08	0.037
Nonylphenol		5.92		218 / 300 277 / 300	218 → 834 225 → 721 277 → 224	2.0	70	3.63	0.15
Diethyl Phtalate		2.42	1173	230 / 355 277 / 355	225 → 6 917 230 → 7 111 277 → 1 260	1.4	80	1.68	0.015
Dibutyl phtalate		4.5	62	230 / 358 275 / 358	225 → 6 817 230 → 6 963 275 → 1 254	1.6	60	3.86	0.016

Material and methods

Absorption and fluorescence spectrophotometer

UV-Visible absorption spectra are recorded on a Eclipse UV-Visible spectrophotometer (Varian). Excitation and emission fluorescence spectra are recorded on a Cary Eclipse Fluorescence spectrophotometer (Varian) with an arc-xenon lamp as excitation source pulsed at 80 Hz.

Laser system and detection device

For laser induced fluorescence measurements, the light source is a Powerlite Precision 9010 (Continuum, Santa Clara, CA) pulsed Nd:YAG pump laser beam at a 10 Hz repetition rate, with a Sunlite EX OPO and FX-1 UV frequency extension system from Continuum, which allows continuous wavelength scanning from 225 nm to 1,750 nm. The available energy in the UV domain ranged from 2 mJ at 225 nm to 10 mJ per pulse at 275 nm [17, 18]. It can be lowered by positioning a divergent lens in the optical path. For our experiments, the energy received by the sample at any wavelength was less than 800 μJ per pulse.

The detection device included a spectrometer and an intensified CCD camera located 50 cm away from the sample cuvette. The fluorescence was collected at 90° angle from the excitation beam and focussed with a f/8 cm lens. The SpectraPro-750i spectrometer (Acton Research Corporation, Acton, MA) had a 750- mm focal length and was equipped with a triple grating turret.

The ICCD-MAX intensified CCD Camera (Princeton instruments, Trenton, NJ) had a 512×512 array optimised for the UV-visible domain. A 0.2 nm/pixel resolution is reached with the 150 g/mm grating. The camera was operated with a ST-133 controller (RS Princeton Instruments, Trenton, NJ) for data acquisition and 16 bit digital conversion. Timing control was achieved with a DG 535 digital delay/pulse generator (Stanford Research System Inc, Sunnywale, CA). The WINSPEC 32-bit Windows software package (Roper Scientific Inc, Trenton, NJ) provided acquisition, display and processing functions.

Chemicals

Phenolic compounds used: 4-n-Nonylphenol: $C_{15}H_{24}O$ (Fluka); Bisphenol A: $C_{15}H_{16}O_2$ (*2,2-bis(4-hydroxyphenyl) propane, 4,4'-isopropylidenediphenol*; Aldrich); 4-Chlorophenol: C_6H_5ClO (Fluka); Phenol: C_6H_6O (*hydroxybenzene*; Sigma).

Phtalate compounds used: Phthalic acid: $C_8H_6O_4$ (*1,2-benzenedicarboxylic acid*; Fluka); Diethyl phthalate (DEP): $C_{12}H_{14}O_4$ (*Phthalic acid di-n-ethyl ester*; Aldrich); Dibutyl phthalate (DBP): $C_{16}H_{22}O_4$ (*Phthalic acid di-n-butyl ester*; Aldrich).

Humic acids (Riedel de Haën), stock solutions were prepared at pH 8 and working solutions were prepared daily.

Dilutions are made in ultrapure water from a Millipore Mro-MQ system. Experiments were carried out under atmospheric conditions at 19 °C.

Experimental results

Absorption and fluorescence properties

For all the studied compounds, the molar extinction coefficient ε has been determined from UV spectrum (absorbance<0.5). The greatest value has been found for bisphenol

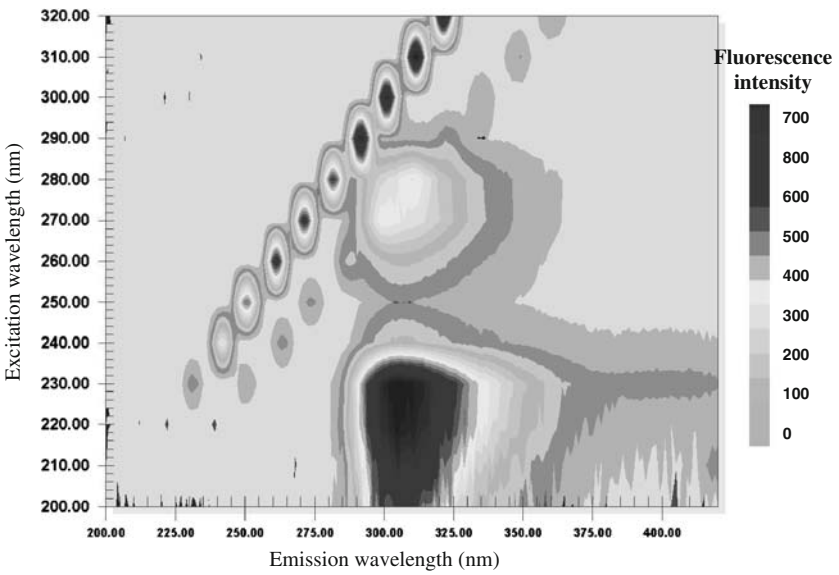


Fig. 1 Excitation/emission matrix of Bisphenol A (7 mg.L^{-1})

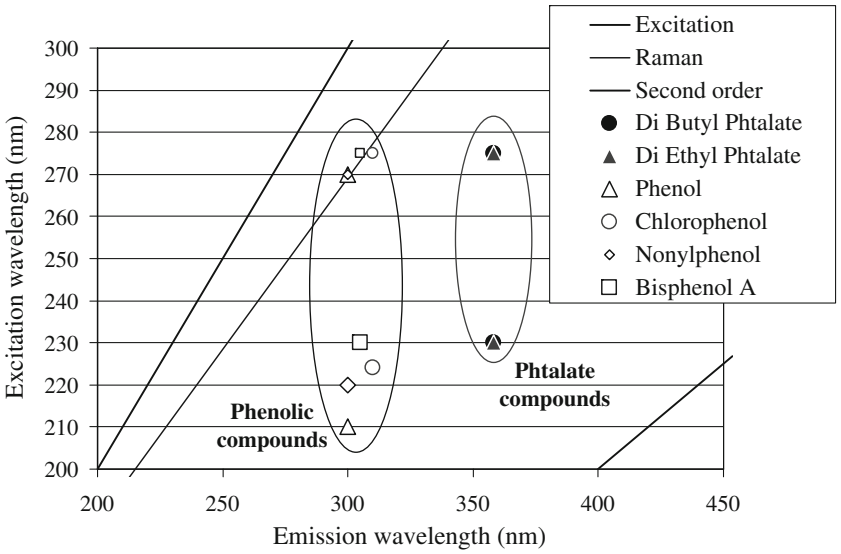


Fig. 2 Excitation/emission maxima of the endocrine disrupting compounds studied

A (ε=13 775 L.cm^{-1}.mol^{-1} at 226 nm) and chlorophenol (ε=13 455 L.cm^{-1}.mol^{-1} at 225 nm). The results are presented in Table 1.

The 3-dimentional fluorescence excitation / emission matrix has been recorded for all the compounds and the example of bisphenol A is presented in Fig. 1 showing a main excitation wavelength at 226 nm, a secondary excitation wavelength at 276 nm and a fluorescence emission maximum at 305 nm. The characteristic excitation/emission couples have been measured for the different EDCs (Table 1) and their locations on the excitation/emission matrix are presented in Fig. 2. One can see clearly that two groups are observed. Compounds from phenolic group present their main excitation wavelengths between 210 nm and 230 nm, a secondary excitation wavelength at 270–275 nm and a fluorescence emission maximum at 300–310 nm. Compounds from phtalate group present their main excitation wavelengths at 230 nm, a secondary excitation wavelength at 275 nm and a fluorescence emission maximum at 360 nm. These characteristics allowed to excite all the EDCs at 225 nm and to identify the group by its fluorescence emission wavelength.

A particular fluorescence spectrum is obtained for phtalic acid (pka$_1$=2.89, pka$_2$=5.51) which exhibits two fluorescence bands at 350 nm and 450 nm (Fig. 3). The 350 nm emission band appears alone at pH=1 and correspond to the protonated form (λ_{EX}=270 nm; λ_{EM}=350 nm); the 450 nm emission band appears alone at pH=8 and correspond to the dissociated form (λ_{EX}=305 nm; λ_{EM}=450 nm). Because phtalic acid has a very low fluorescence response in water the other parameters could not be determined.

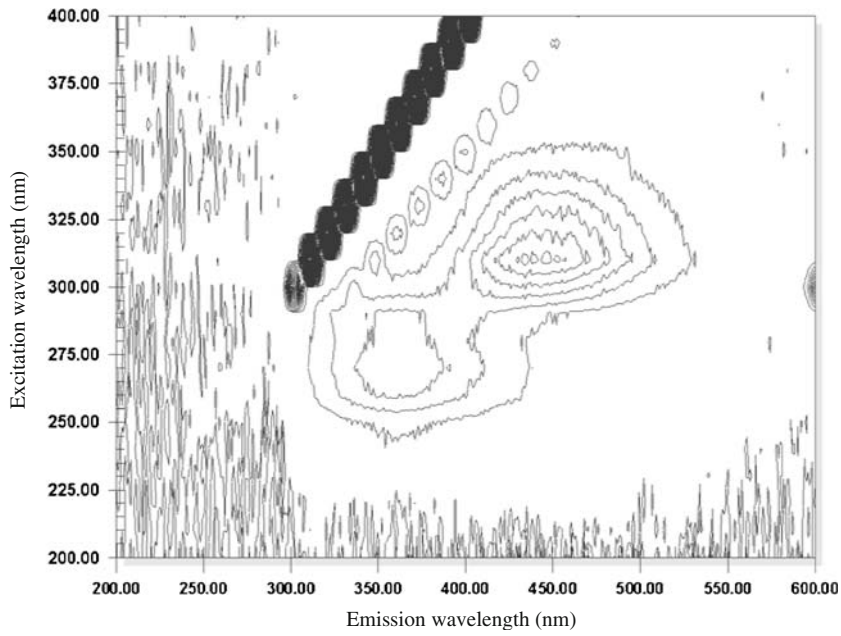


Fig. 3 Excitation/emission matrix of phtalic acid (680 mg.L^{-1})

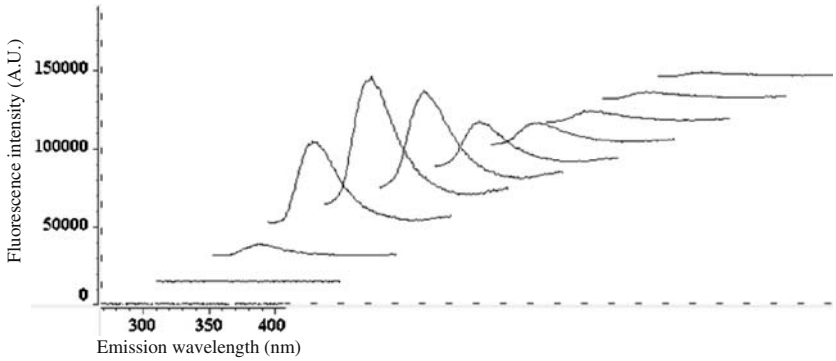


Fig. 4 Time resolved fluorescence of nonylphenol. Each spectrum his recorded with a time gate of 2 ns. A time delay of 2 ns separates the spectra

Fluorescence lifetime

The fluorescence lifetime of the compounds has been studied by laser induced fluorescence. The fluorescence spectra have been recorded in temporal resolution mode with a gate time of 2 ns and a time shift of 2 ns between each spectrum (Fig. 4, example of the nonylphenol). The fluorescence decay versus the time follows the Relation 1. The fluorescence lifetimes have been determined by linearization of the fluorescence temporal decay (Relation 2) where the slope of the linear domain is equal to -1/T (Fig. 5, example of the bisphenol A). The fluorescence lifetimes found are short (3 ns to 4 ns) and not significantly different for the different compounds studied. Only the diethyl-phtalate has a shorter lifetime of 1.7 ns. The other detailed results for the studied compounds are presented in Table 1.

$$I_t = I_0 \cdot \left(e^{-\frac{t}{\tau}}\right) \quad (1)$$

$$Ln(I_t) = Ln(I_0) - \frac{t}{\tau} \quad (2)$$

Fluorescence quantum yield

The fluorescence quantum yield has been determined from absorbance and fluorescence measurements. For each compound, several solutions with increasing concentrations have been prepared. For each solution, the absorbance has been measured at 225 nm. Then the fluorescence intensity has been measured for the same solutions (absorbance lower than 0.1 to avoid inner filter effects), at the excitation wavelength of 225 nm. For each compound, the fluorescence intensity increases linearly with the absorption at low absorbance values. In agreement with Relation 3 (where I_F is the integrated fluorescence, Φ_F the fluorescence quantum yield, A the absorbance at 225 nm and I_{exc} the intensity of the excitation), the variations of I_F versus $(1–10^{-A})$ are linear and the slope is equal to $\Phi_F I_{exc}$ (Fig. 6). By reference to phenol (Φ=0.22 in water [19]) the slope ratio (Relation 4) permits to calculate the fluorescence quantum yield of the compound.

$$I_F = \Phi_F I_{exc}\left(1 - 10^{-A}\right) \quad (3)$$

$$\frac{Slope_{\ X} \cdot \Phi_{Phénol}}{Slope_{\ Phénol}} = \Phi_X \quad (4)$$

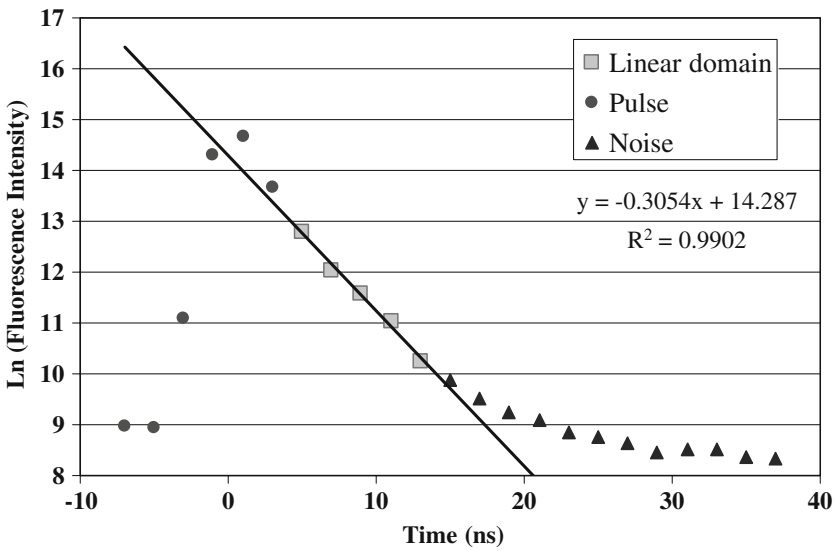


Fig. 5 Neperian logarithm of the fluorescence intensity of Bisphenol A versus the time. The maximum of the laser pulse intensity correspond to t=0

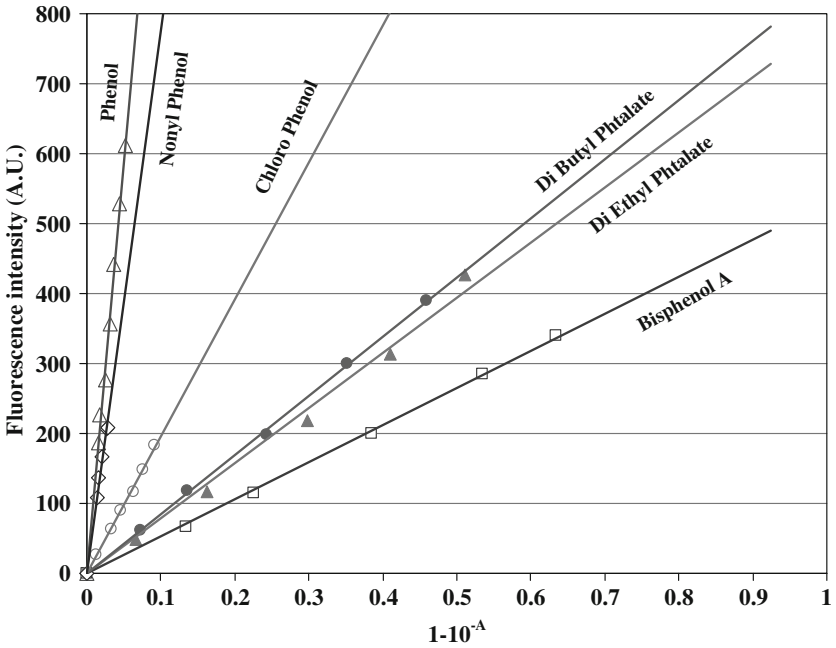


Fig. 6 Variation of the fluorescence intensity versus $(1–10^{-Absorbance})$

The results are presented in Table 1. It is observed that the phenol has the highest quantum yield (Φ=0.22), close to that of the nonylphénol (Φ=0.15); the diethyl-phthalate and the dibutyl-phthalate have both a medium value and the Bisphenol A has the weakest one (Φ=0.010).

Direct analysis by laser induced fluorescence

As tap water and environmental controls are important problems, classical analytical methods have been developed for ECDs determination. These protocols used a solvent or SPE cartridges extraction step followed by a gas or liquid chromatographic separation and a mass spectrometry detection [20–24]; however these methods are time consuming and present some risk of sample contamination through flask storage. Therefore, direct analysis may be a useful tool. For this purpose we used a pulsed YAG laser with an Optical Parametric Oscillator as a high energy excitation source and an ICCD camera for highly sensitive fluorescence detection [15, 16]. The example of Bisphenol A is presented in Fig. 7.

The limit of detection has been calculated with the Relation 5, where σ is the standard deviation of ten blank intensity measurements, H the fluorescence intensity of the compound solution minus the fluorescence of the blank and C the concentration. All intensity measurements have been

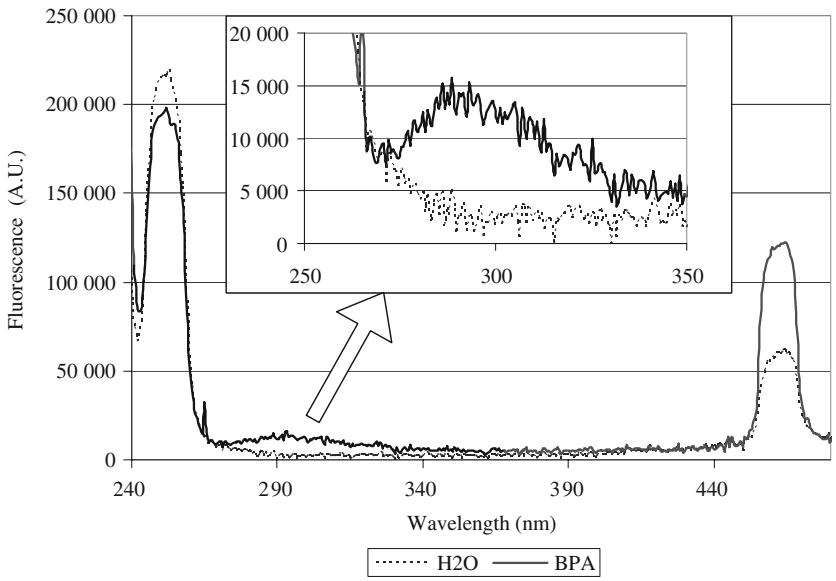


Fig. 7 Fluorescence spectra of Bisphenol A in tap water $(10\ \mu g.L^{-1})$

averaged on 10 nm around the emission maximum of the compound under study.

$$LD = 3\sigma\frac{C}{H} \quad (5)$$

The detection limits obtained are low (between 2 μg L^{-1} for the Bisphenol A and 0.35 μg.L^{-1} for the Chlorophenol). The detailed results are presented in Table 1 for the studied compounds. In the case of the Bisphenol A, for example, the detection limit obtained is lower than the known environmental recommended PNEC limit [25–27] which is equal to 64 μg.L^{-1} [10], meaning that the water quality can be easily monitored by this method. For the other studied compounds, the detection limit is always lower than the PNEC value when it is reported (Table 1).

We also studied ECDs in raw water containing humic acids (1 mg.L^{-1}). Humic acids present a fluorescence maximum at 470 nm but their emission band is wide and the fluorescence intensity, very strong at this concentration, can be observed even at 335 nm. Consequently, the detection limits obtained in raw water are about ten to 20 times highest than in tap water.

Discussion

One can note that the nonylphenol has a rather high fluorescence quantum yield of 0.15 but a low absorbance (ε= 720 L.cm^{-1}.mol^{-1}), which leads to a detection limit of 2 μg.L^{-1} by laser induced fluorescence. For the Bisphenol A, the detection limit is similar (2 μg.L^{-1}) even with a lower fluorescence quantum yield (Φ=0.010) which is compensated by a higher absorbance (ε=13 760 L.cm^{-1}.mol^{-1}). In the case of the chlorophenol, the conjunction of a medium fluorescence quantum yield (Φ=0.037) and a high absorbance (ε=13 450 L.cm^{-1}.mol^{-1}) allowed to obtain the best detection limit of 0.35 μg.L^{-1}.

A multilinear regression permits to obtain the following equation between the detection limit (DL), versus the molar extinction coefficient (ε) and the fluorescence quantum yield (Φ).

$$DL = 1.243 + 102.55\,10^{-6} \times \varepsilon + 8.04 \times \Phi + 5.23\,10^{-3} \times \varepsilon \times \Phi$$

It shows that increasing the fluorescence quantum yield or the molar extinction coefficient have both an important effect and strongly decreases the detection limit. Moreover, the interaction between ε and Φ is highly significant and expresses that the conjunction of a high ε value and a high Φ value induces an important decrease of the DL. However, for a low ε value, whatever is the Φ value, the DL remains high, without significant variations.

Conclusion

This study leads to a better knowledge of the spectroscopic properties of some ECDs, particularly fluorescence lifetime and fluorescence quantum yield.

For tap water, low limits of detection can be reached by direct analysis. In every case, they are lower than the PNEC environmental limits. The analysis of these compounds in raw water, where they are present at trace level, is however more difficult to realize. As humic acids fluorescence lifetimes are about 4 ns, the short lifetime of ECDs studied does not allow to use a temporal shift to eliminate the fluorescence of humic acids.

From a general point of view, direct determination or screening in tap water using laser induced fluorescence, may be interesting for many compounds even with very low fluorescence quantum yields. For raw water (containing humic substances) the direct analysis of compounds requires a fluorescence lifetime about 15 ns longer than those of the EDCs studied. Nevertheless, the absence of analyte at a concentration higher than the calculated LD for this medium can be assumed by direct screening.

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