Fluorescence techniques for the determination of polycyclic aromatic hydrocarbons in marine environment: an overview

J.J. Santana Rodríguez* and C. Padrón Sanz

Department of Chemistry, Faculty of Marine Sciences, University of Las Palmas de G.C., 35017 Las Palmas de G.C., Spain

Polycyclic Aromatic Hydrocarbons (PAHs) are compounds of great environmental interest because of their potential carcinogenic and mutagenic activity and their frequent occurrence in the environment, above all in the marine environment. Therefore it is an important to establish simple, sensitive and reliable methods for the determination of these compounds. In this work we report an overview on the application of fluorescence techniques to the study and determination of PAHs in marine samples: water, sediments and organisms. Conventional fluorescence spectroscopy, synchronous fluorescence spectroscopy, Shpol´skii fluorescence spectroscopy and high performance liquid chromatography (HPLC) with fluorescence detection constitute the most interesting analytical techniques for the determination of these pollutants.

In the last few years, there has been an increasing interest in the types and concentrations of organic compounds present in marine environment. Among them, notable interest has been shown in PAHs pollutants, as demonstrated by the high number of recent papers in this field.

PAHs are a class of organic compounds, which are included in the wider family of polycyclic aromatic compounds (PACs). These compounds have shown carcinogenic and/or mutagenic activity in laboratory experiments with animals. PACs are generally formed during incomplete combustion or pyrolysis of organic matter occurring in a variety of natural processes or human activities. Consequently, PAHs are ubiquitous pollutants, which are present in all environmental components. Specifically, the presence of PAHs has been reported in marine environment: sea water [5-9, 29-33], sediments [2,3,35-51], and plankton, seaweed and filter feeding organisms [52-71].

These pollutants can enter in the oceans by many routes, including petroleum spills, runoff from roads, sewage, effluents from industrial processes, and fallout from the atmosphere. Sixteen of them are included in the list of priority pollutants by US Environmental Protection Agency (Tab. I) [1]. That is why monitoring their presence and persistence is the main environmental problem, and it is essential to find analytical methods capable to identify and quantify these pollutants in the environment.

In the last few years, important advances have been made in improving existing analytical methods and developing new techniques for analysis of PAHs in marine environment, many of them appearing in scientific and technical journals and symposium proceedings.

From the various molecular spectrometric techniques compared, one singularly important trend can be seen: the absorbency and fluorescence spectra of the PACs contain far more information than those for the other classes of compounds. This is especially true for the PAHs, where many

Table I. List of priority pollutants PAHs considered by EPA (Environmental Protection Agency), their carcinogenity and ocurrence.[a]

Hydrocarbon	*Molecular formula*	*Carcinogenicity[b]*	*Occurrence[c]*
Naphthalene	$C_{10}H_8$	–	E
Acenaphtene	$C_{12}H_{10}$	–	E
Acenaphtylene	$C_{12}H_8$	–	F
Fluorene	$C_{13}H_{10}$	–	E,F,S
Phenantrene	$C_{14}H_{10}$	–	E,F,S
Anthracene	$C_{14}H_{10}$	–	E,F,S
Fluoranthene	$C_{16}H_{10}$	–	E,F,S
Pyrene	$C_{16}H_{10}$	–	E,F,S
Benzo(a)anthracene	$C_{18}H_{12}$	+	E,F,S
Chrysene	$C_{18}H_{12}$	– +	E,F,S
Benzo(b)fluoranthene	$C_{20}H_{12}$	++	E,F
Benzo(k)fluoranthene	$C_{20}H_{12}$	–	E,F
Benzo(a)pyrene	$C_{20}H_{12}$	+++	E,F,S
Dibenzo(a,h)anthracene	$C_{22}H_{14}$	+++	E,S
Benzo(g,h,i)perylene	$C_{22}H_{12}$	–	E,F,S
Indeno(1,2,3-cd)pyrene	$C_{22}H_{12}$	+	E,F

a) Reference [1]
b) +++ or ++ = strongly carcinogen; + = carcinogen; – = not carcinogen
c) E = Environment (water, air, tobacco smoke, gasoline and diesel exhaust); F = Foods; S = Curing smoke

Article available at http://analusis.edpsciences.org or http://dx.doi.org/10.1051/analusis:2000280710

structures have spectra with a dozen or so maxima. These maxima as well as the intervening minima, form a unique characteristic pattern of wavelengths and intensities. The pi electron system also causes the PAHs to have a different fluorescence and phosphorescence behaviour. Especially for the PAHs, the electronic transitions are determined by the size and shape of the compounds. Electronically, the PAHs are simple molecules whose spectra are only determined by the molecular resonance structures. Isomeric PAHs differ in the locations of electronic density, because the shape of the PAHs are different, and in some cases they can even differ in their numbers of aromatic rings.

Because of this fundamental reason and of the high sensitivity and selectivity of the chromatographic techniques, HPLC has been used predominantly. Single-wavelength UV absorbency or fluorescence detectors have been applied for PAHs analysis. These are used to detect PAHs because the pi electron systems of PACs determine the energies of the transition in the electronic spectra.

Between both spectroscopic techniques above mentioned, the most sensitive is fluorescence spectroscopy because of the direct measurement of the emitted light intensity with little background or interference. The present overview is centred on the methodologies which apply and/or include fluorescence in the analysis of PAHs in marine environment.

Fluorescence techniques

Conventional fluorescence spectroscopy

Conventional fluorescence spectroscopy involves generating an emission spectrum by scanning the emission wavelength, λ_{em} while the sample is irradiated at a single excitation wavelength, λ_{ex}. Similarly, an excitation spectrum is obtained by scanning the excitation wavelength while recording the emission signal at a single wavelength. Furthermore, spectrofluorimetry is a very simple method, which offers generally very low detection limits and can be used with conventional instrumentation.

This technique is very useful in quantitative analysis of PAHs because of its high sensitivity, selectivity, swiftness and relatively low cost. Moreover, it can be applied to determine PAHs in many environmental marine samples.

The use of fluorescence spectroscopy as a detection technique to determine PAHs in marine sediments was applied by Vandermeulen *et al*. [2]. After *Amoco Cadiz* accident the immediate behaviour and toxicity of freshly spilled crude oil under prevailing spill conditions in an inshore marine environment was examined. Authors simulated weathering studies because weathering states of oil ranging from sheen oil to mousse have significant effects during oil-water and oil-sediment interaction.

Observation of oiled sandy beaches suggest two mechanisms of beach contamination: general penetration and contamination of beach substrate by films of sheen oil (probably partially emulsified), and secondly the burial of discrete layers of mousse. Depending on the timing of oiling with respect to beach dynamics, large amounts of stranded oil can be accommodated within beach sediments. The results of this investigation suggested that there may be some increase in toxicity with weathering, because PAHs become available for later long-term release.

In the case of another kind of marine samples, Picer and Hocenski [3] described a relatively simple, rapid and quite reliable procedure, based on the fluorescence of PAHs, for the estimation of petroleum hydrocarbons in organisms and marine sediments. In this study lipophilic material was extracted from the samples with n-hexane or n-pentane, concentrated to approximately 1 ml, and benzene mixtures. The fluorescent material was cleaned by using a deactivated alumina column, and for the evaluation of the quenching material, the standard addition method was applied. The aim of the work was to improve the possibilities of estimating crude oil pollution of sediment and biota samples by using the standard addition method.

Applying fluorescence on solid organic substrate Vo-Dinh and White [4], investigated a simple technique based on sensitized luminescence for detecting trace amounts of polynuclear aromatic compounds. They used anthracene as the sensitizer designed to absorb excitation energy and funnel it to guest analyte compounds spotted on anthracene treated filter paper. Perylene, benzo(a)pyrene and fluoranthene mixtures were analyzed. Results indicated that anthracene can improve the fluorescence signal of perylene and benzo(a)pyrene. No fluorescence sensitization was observed for other compounds, such as fluoranthene.

As showed above, fluorescence spectroscopy is a technique applicable to the determination of PAHs in solid phases as sediments or marine organism tissues. It exists also a large number of works and publications in which this technique is applied to determine PAHs in marine water samples.

Vapu Tervo [5] determined the total amount of petroleum hydrocarbons (oil) from 52 seawater samples collected at 19 stations in the Gulf of Finland. The samples were analysed ashore by fluorescence spectroscopy. The mean concentrations obtained for “light oil” were 1.6-7.3 μg/l and for “heavy oil” 0.5-1.6 μg/l in the whole investigation area.

Law [6] also applied this technique to samples of water, sediment and oil following the wreck of the *Amoco Cadiz* in 1978. Samples were collected between April and June from the Brittany coast and western English Channel and were analysed for petroleum hydrocarbons by means of fluorescence spectroscopy, gas-liquid chromatography and gas chromatography-mass spectrometry. The concentrations of hydrocarbons found in these samples, 2-200 μg/l, were similar to those found in previous oil-spill studies.

As well, after the oil spill accident at *Mitsubishi Oil Refinery* in Kurashiki city, Ochi and Okaichi [7] measured oil residues by fluorescence spectroscopy in sea water of

Bisan Seto. In another study, achieved by Østgaard and Jensen [8], the fluorescence of carefully prepared solutions of Ekofisk crude oil in sea water was investigated, directly in the water phase, it was concluded that direct fluorescence spectroscopy constitutes a very simple and rapid method for monitoring aqueous petroleum solutions. In this work, significant fluorescence in the 290-460 nm range for oil solutions well below the concentration level of 10 ppb was observed. It was also established that, on the basis of spectra of the single components, the influence of pH on these, and data from gas chromatography/mass spectrometry, the fluorescence at 335 nm (excitation wavelength 230 nm) was dominated by petroleum naphthalenes, while the fluorescence at 300 nm (excitation wavelength 265 nm) could be ascribed to the phenols of the crude oil.

In analytical methods currently used for assaying PAHs in aqeuous media preliminary extraction of the water with organic solvents is required to remove interfering substances. With the use of micellar media, a system which enables the extraction and preconcentration of analytes, these organic solvents can be replaced, allowing a faster and reduced cost analysis. Moreover, micellar media can be used to enhance sensitivity and selectivity of many fluorimetric determinations. Thus, Santana *et al*. [9] studied the use of different micellar media, as a way to improve the spectrofluorimetric determination of benzo(a)pyrene (BaP), compound of great toxicological interest and for its significance in pollution studies. They found that BaP fluorescence intensity suffers important changes in cationic and neutral micellar media and showed the greatest increase in the presence of Triton X-100. The method was applied to determination of BaP in seawater samples enriched with this hydrocarbon.

Another variant, also applied to determination of PAHs in marine environmental samples consists in laser induced fluorescence spectroscopy. Uebel *et al*. [10] applied this methodology to the analysis of PAHs and other pollutants in marine environmental samples. They used a frequency doubled dye laser as an excitation light-source, recording the fluorescence signal and measuring each contaminant spectra in distilled water, artificial and natural seawater. Firstly the intermediate stages of phytoplankton and their breakdown-products were spectroscopically recognizable using fluorescence. Modifications of the fluorescence spectra during the ageing and dying of phytoplankton were found. Phytoplankton which is subjet to sudden death display spectra differing from those of slowly dying organisms, *e.g.* the shift of the chlorophyll maximum to shorter wavelegths was measured.

Synchronous fluorescence spectroscopy

Even though conventional fluorescence spectroscopy has an excellent sensitiviy in determining trace organic compounds, its aplication to the simultaneous determination of several fluorescent compounds in mixtures, is more limited, moreover when these compounds have a high structural similarity. This is due to the fact that the individual spectra of the compounds in the mixture can overlap. This problem can be resolved using synchronous fluorescence spectroscopy, which was firstly introduced by Lloyd [11]. Conventional fluorescence involves generating an emission/excitation spectrum by scanning the emission/excitation wavelength, while the sample is irradiated at a constant excitation/emission wavelength. Another possibility is to vary simultaneously λ_{ex} and λ_{em}. This technique has several variants, depending on the scan-rates of the two monochromators. When the scan-rate is constant for both monochromators, and therefore a constant wavelength interval, $\Delta\lambda$, is kept between λ_{em} and λ_{ex}, the technique is known as synchronous excitation fluorescence spectroscopy.

Since conventional emission and excitation fluorescence spectroscopy from mixed PAHs-solutions show great interference, the synchronous fluorescence technique can simplify the spectra and therefore some mixtures of PAHs can be quantified. In this way, Santana *et al*. [12] studied the interactions between benzo(a)pyrene and perylene with different kinds of surfactants. They developed a synchronous spectrofluorimetric method to determine simultaneously BaP and perylene using Triton X-100 as micellar medium. The limits of detection obtained were 0.27 and 0.3 ng/ml, respectively. The method was applied to the analysis of these compounds in seawater samples.

Other investigations in this field has been developed studying other PAHs such as benzo(g,h,i)perylene, chrysene, dibenzo(a,c)anthracene and coronene [13] as well as mixtures of them, in presence of anionic, cationic and non ionic surfactants. Bermejo *et al*. [14] established a method for the simultaneous determination of perylene and benzo (g,h,i)perylene using hexadecyltrimethylammonium bromide (HDTAB) micellar medium and synchronous fluorescence, obtaining limits of detection of 0.12 and 0.21 ng/ml for both compounds respectively. The method was also applied to the determination of such PAHs added to sea water samples with acceptable results, which demonstrates that synchronous fluorescence spectroscopy can be used as a rapid, simple and sensitive method for the determination of perylene and benzo(g,h,i)perilene in marine water samples.

Santana *et al*. [15] studied the spectrofluorimetric characteristics of chrysene, perylene, dibenzo(a,c)anthracene and coronene in aqueous medium and in the presence of anionic, cationic and non ionic surfactants, employed specifically to enhance the selectivity and sensitivity of the fluorescence determinations of these compounds. Using hexadecyltrimethylammonium bromide as surfactant and synchronous fluorescence, these authors established two methods for the analysis of binary and ternary mixtures of these PAHs. In the first one dibenzo(a,c)anthracene and coronene were determined simultaneously employing $\Delta\lambda$ = 89 nm, with limits of detection of 0.20 and 0.22 ng/ml, respectively. In the second method, three different values of $\Delta\lambda$ were utilized for the simultaneous determination of chrysene, perylene and coronene (41, 3 and 140 nm respectively). Limits of detection were 0.17 ng/ml for chrysene, 0.13 ng/ml for perylene and 0.14 ng/ml for coronene. Satisfactory recovery percentages were found when both methods were applied to

the analysis of binary and ternary mixtures of these PAHs added to seawater.

As well, Böckelen and Niessner [16] probed the application of several non-ionic surfactants for the extraction and enrichment of PAHs from aqueous media. Developing a spectroscopic method for the simultaneous detection of PAH-mixtures by synchronous fluorescence in micellar medium, they obtained recoveries up to 100 % with limits of detection of 6.8 and 2.6 ng/l for benzo(k)fluoranthene and benzo(a)pyrene, respectively.

Synchronous fluorescence spectroscopy has also been applied to determine PAHs in other environmental samples such as marine organisms. For example, Ariese *et al*. [17] determined the uptake of PAHs by fish, screening the gall bladder bile for PAHs metabolites. They proposed synchronous fluorescence spectroscopy as a rapid screening technique for the determination of conjugated 1-hydroxy pyrene, wich is a major metabolite in bile of fish exposed to PAHs polluted sediment. The technique was applied to a mesocosm study in which the uptake of PAHs by flounder (*Platichthys flesus*) from polluted sediment was investigated. In a subsequent study, the method was applied to the southern North Sea and in Dutch coastal and inshore water [18].

Conventional and synchronous fluorescence spectroscopy, together with solid phase spectrofluorimetry, are adequate techniques for determination of PAHs. The advantages of the latter one are that only a little amount of a convenient solid support is needed for the preconcentration of the analytes presenting inherent fluorescence, and that fluorescence measurements can be carried out directly in the solid phase. Applying these techniques to waters from different sources (tap, natural, waste and sea water), Vilchez *et al.* [19] determined by solid-phase spectrofluorimetry the content in benzo(a)pyrene, benzo(a)anthracene and pyrene, wich exhibit native fluorescence in solution at trace levels. The relative fluorescence intensity was measured with these PAHs fixed on Sephadex G-25 gel after packing the gel beads in a 1 mm silica cell. By recording the synchronous spectra at different values of $\Delta\lambda$, benzo(a)pyrene, benzo(a)anthracene and pyrene can be simultaneously determined in the presence of other PAHs.

Shpol'skii fluorescence spectroscopy

Shpol'skii spectroscopy is especially suited for the qualitative analysis of PAHs at trace levels as it combines the sensitivity inherent to fluorescence methods with the specific information that can be obtained in infrared spectroscopy. It makes use of frozen n-alkane matrices at cryogenic temperatures to considerably reduce band broadening which is the cause of the limited identification power of room temperature fluorescence. The PAHs occupy substitutional sites in the n-alkane crystal resulting in largely identical surroundings. The appearance of the Shpol'skii spectrum may vary if different n-alkanes are employed, because the fit of the PAH within the crystal is critical. A Shpol'skii spectrum consists of a number of narrow lines with a full width at half maximum of 0.1-0.01 nm. These lines are suitable for identification purposes because they form a fingerprint of the individual PAHs.

Ewald *et al*. [20] applied the technique to the study of marine sediments and demonstrated that high resolution spectrofluorimetry at 4.2 K in n-alkane matrices can be used to identify polycyclic aromatic hydrocarbons derived from triterpene, which occur in the organic matter of marine and terrestrial sediments.

Garrigues *et al.* [21-23] used this technique for the analysis of several methylated-PAHs series in marine samples: organic material, sediments and crude oils. The analytical results were compared with those reported by other methodologies and were found to be in quite good agreement, thus demonstrating the reliability of high resolution Shpol'skii spectrofluorimetry. Also, Hofstraat *et al*. [24] determined PAHs in harbour sediments by means of Shpol'skii fluorimetry and showed it was an appropriate analytical method for the quantitative and qualitative determination of PAHs in such samples. Moreover, they concluded that this technique yields low limits of detection comparable to those obtained by a standard procedure based on HPLC with fluorimetric detection.

Applied to other marine samples, Ariese *et al*. [25] investigated the applicability of high-resolution Shpol'skii spectrofluorimetry to the direct analysis of polycyclic aromatic hydrocarbons in tern and mussel samples. The sensitivity of the measurements suffered considerably from the large amounts of interfering substances (e.g. fatty components) in the crude extracts, resulting in a poor-quality Shpol'skii matrix and a high sample absorbency. Nevertheless, after a thorough study of these limiting factors, optimum conditions could be defined and a number of PAHs were detected directly without any sample clean-up.

Shpol'skii spectroscopy cannot be used for on-line detection in HPLC, since the solid matrix precludes compatibility with flow systems. Therefore Shpol'skii fluorimetry was applied by Mastenbroek *et al.* [26] as an independent identification method to the upgrade routine HPLC analysis of Polycyclic Aromatic Hydrocarbons. HPLC combined with fluorescence detection is routinely used in the Dutch Water Quality Survey to determine the PAHs content of marine sediment samples. In this study, this methodology is utilized to ascertain the identity and the purity of the peaks in the chromatograms by collecting several eluting fractions and using subsequent spectroscopic analysis. They found that low temperature Shpol'skii technique provides high-resolution fluorescence spectra of PAHs that can serve as fingerprints. Thus, important information concerning peak purity was obtained and the number of components identified was roughly doubled.

Kozin *et al*. [27] also applied high-resolution low temperature molecular luminescence technique, to the analysis of various complex environmental matrices to get isomer specific information for a wide range of PAHs and their derivatives. Using conventional lamp excitation and fluorescence and phosphorescence detection, they determined

parent priority PAHs, six-membered ring compounds, and methyl-substituted PAHs in extracts of sediment, muscles from finfish and other samples.

Hofstraat *et al* [28] employed laser induced Shpol'skii fluorimetry as a variant of the previous technique. This methodology is also applicable to the analysis of PAHs in marine environmental samples, especially the PAHs content in biotic samples. The authors showed that Shpol'skii technique can be used successfully as an independent reference method for both qualitative and quantitative confirmation of conventionally used determination methods.

Fluorescence detection in liquid chromatography

Usually, liquid chromatography using fluorescence detection (LC/fluorescence) and gas chromatography coupled to mass spectrometry (GC/MS) are the most powerful techniques for monitoring PAHs. Nevertheless, an advantage of LC/fluorescence is its ability to measure some PAHs isomers that cannot be easily quantified by GC/MS. The large and/or nonvolatile polycyclic aromatic compounds (PACs) cannot be analyzed by GC because either they will not elute or, if they do, the peaks will be unacceptably broad. In addition, some PACs are thermally unstable and decompose or rearrange pyrolytically to other structures. Because of its excellent separation and detection performance, LC has been specified as the method of choice by the U.S. EPA for the analyses of aqueous effluents for the determination of PAHs.

Water

The determination of PAHs in water and wastewater is an area which has attracted the interest of a large number of researchers for many years. It was observed a significant evolution in the techniques applied to PAHs determination in water. Prior to 1964, researchers tend to employ the separation techniques of solvent extraction, classical column liquid chromatography, and thin layer chromatography with UV absorption or fluorescence detection. Since 1980 the predominant analysis method for water samples was reverse phase high performance liquid chromatography (HPLC) with fluorescence detection. Several papers addressed proper column selection, analysis conditions, and detection. The most commonly used and most rigorous approach for trace concentration of PAHs was the methodology proposed by EPA for PAHs extraction. Currently, the use of micellar media as an extraction and preconcentration system is applied as an alternative. This methodology allows an important improvement with a reduced cost and time of analysis.

Lee *et al.* [29] determined hydrocarbons content and its evolution in several different kind of environmental samples. *Prudhoe* crude oil enriched with a number of polycyclic aromatic hydrocarbons was added as a dispersion to a controlled ecosystem enclosure suspended in *Saanich Inlet*, Canada. Concentrations of various aromatic compounds were measured by passing 5 µl of a methanol solution through an assembled liquid chromatograph equipped with a fluorescence detector. PAHs were determined in water, zooplankton, oysters, and bottom sediments. Initial water concentrations of the lower weight aromatics, naphthalenes and anthracene, were 10-20 µg/l, whereas the initial concentrations of benzo(a)pyrene, benz(a)anthracene, and fluoranthene ranged from 1 to 6 µg/l. These concentrations decrease at an exponential rate because of evaporation, photochemical oxidation, microbial degradation, and sedimentation. Only naphthalenes were significantly degraded by microbes with removal by this process of up to 5 % per day from the water. Sedimentation and photochemical oxidation were responsible for the decrease in concentrations of the higher molecular weight aromatic compounds.

The determination of PAHs in aqueous samples is rather difficult, as their concentration in water is extremely low due to their poor solubility. In order to detect the PAHs at low levels in water, a concentration step is generally required. Use of surfactants allows the extraction and preconcentration in only one step. Micelles formed by surfactants have the capability of solubilizing organic compounds in water. Using this procedure, substrates can be extracted from aqueous media in a much smaller volume nearly formed by the surfactant. In addition, aqueous solutions of surfactants have been used as mobile phases in liquid chromatography, and although this kind of mobile phase was primarily used in column chromatography and thin layer chromatography, they are now widely utilized in HPLC. One of these surfactants is Brij-35 [31,32].

In the same way, Brouwer *et al.* [32] used liquid chromatography method with fluorescence and diode-array UV detection for the trace-level determination of the sixteen EPA-priority PAHs. The system was used for the analysis of surface water samples. The procedure involved on-line micelle-mediated preconcentration on selective sorbents. In this study, the authors used the solubilizing properties of several ionic and non-ionic surfactants, to solve the problems arising from the low solubility of PAHs in water and their sorption to surfaces, and set up an on-line trace enrichment LC system with fluorescence detection. Using Brij-35 as surfactant, unwanted adsorption of the analytes on inner walls or surfaces was prevented. The limits of detection obtained were at the low to sub-ng/l level.

In a study performed by Sicilia *et al*. [33], a new approximation in the *cloud-point extraction* methodology was applied to PAHs determination in environmental samples. It was based on the use of anionic surfactants in acidic media, combined with LC/fluorescence detection. For this study, the authors considered previous works in which it was shown that anionic surfactants were separated in two isotrophic phases in acid medium at room temperature. Among others, sodium dodecane sulfonic acid (SDSA) was employed as the surfactant. It has two important advantages over non ionic surfactants, which are generally used in the cloud-point methodology for the extraction of PAHs mixtures [34]. First, it exhibits low fluorescence intensity and absorbance at the

detection wavelength of PAHs (due to absence of aromatic groups in the surfactant molecule) and second, it is characterized by a short retention time (due to its polar character).

Sediments

Dunn [35] performed a very complete investigation concerning PAHs concentration in several marine samples, by trying to determine the relationships existing between the levels of a range of PAH isomers in three different kinds of marine samples: sediments, bivalve molluscs and algae. For this reason, he applied a method based on reversed phase liquid chromatography and fluorescence detection.

Also, Obana *et al.* [36] studied some PAHs (benzo(a)pyrene, dibenzo(a,h)anthracene, and 3-metylcholanthrene), which are carcinogens for mammals after in vivo hydroxylation by mixed function oxidases. Compared to other separation techniques, the development of HPLC has permitted to analyze PAHs with good separation and high sensitivity, and to simplify the pre-treatment processes. In the study, ten PAHs were determined in sediments, oysters, and wakame seaweeds by HPLC with a fluorescence detector.

This methodology was also applied by Smith *et al.* [37] to the determination of PAHs in sediments, seawater and clams from Green Island, the most visited coral island of the Great Barrier Reef in Australia. Results showed that only sediments near powerboat moorings were found to contain low, but measurable amounts of several different PAHs, in contrast to the baseline amounts found at other locations. The presence of several PAHs at measurable levels strongly suggests that their origin was due to fuel spillage and/or exhaust emissions.

In an investigation of marine sediments from the Adriatic Sea, Guzzella and Paolis [38] evaluated PAHs contamination. During a naval cruise from Trieste to Bari they collected thirty two samples and determined PAHs in the fine sediment fraction. The quantification of PAHs content in the samples was performed also with HPLC and fluorescence spectroscopy.

Beltrán *et al.* [39] applied also a HPLC method with fluorescence detection to the determination of the sixteen PAHs considered as the most pollutants by EPA in reference to marine sediments (HS-3, NRCC). The method consisted on HPLC determination of PAHs using isocratic conditions, with spectrofluorimetric detection and programmation of wavelengths.

The organic contaminant analyses in the environment require complex procedures with several steps, such as extraction, purification and quantification. In the case of solid samples, the extraction is often performed by reflux of organic solvent. This method is long (several hours) and solvent-consuming (several hundreds of mL). Microwave-assisted solvent extraction (MASE), represents an interesting alternative method for organic contaminants extraction.

As an example of application, Kay *et al.* [45] developed a MASE technique for the extraction of PAHs in marine sediments. Optimum conditions for this technique were obtained by using the mixed-level orthogonal array design procedure. A comparison between the Soxhlet extraction and MASE methods showed that although both techniques gave comparable results on certified reference materials (HS-2 and HS-6), the MASE technique allows one to use less solvent and it is also time-saving and cost-effective, without affecting the extraction efficiency. The optimum MASE technique was coupled to two analytical techniques: HPLC with both UV and fluorescent detectors as in other previous studies [46-50], and GC-MS for the qualitative and quantitative determination of PAHs in the certified reference materials and real samples (marine sediments).

Another methodology focussed on microwave assisted extraction was applied by Letellier *et al.* [51] and used to extract PAHs from environmental matrices. The procedure was validated on marine sediment, the standard reference material (SRM 1941a). The concentrations obtained by this method were in agreement with the certified values and the concentrations measured using Soxhlet extraction.

Organisms

A large number of papers related with the application of HPLC with fluorescence detection to the PAHs analysis in marine organisms has been published [52-61]. Among them, crustaceans [56] are the most widely investigated. Indeed, they can concentrate a high level of PAHs probably because of the absence of aryl hydrocarbon hydroxylase in these species. In fact, scientists of several specialties have proposed the use of indigeneous bivalve molluscs in order to serve as biocontrollers of the detection and quantification of environmental pollutants, including carcinogens. Due to the fact that they are permanent inhabitants in specific locations and have a tendency to concentrate environmental contaminants, bivalves have been widely investigated for such studies.

In this line of investigation, Hanus *et al.* [62] described an HPLC procedure for the determination of thirteen PAH compounds in oysters at the ppb level. Recoveries obtained from spiked samples were generally close to 80 %. In a particular investigation, Mix and Schaffer [63] focussed on the determination of PAHs present in clams of the Coos Bay in Oregon (USA). In such a study, the concentrations of benzo(a)pyrene were measured in clam populations from four different intermareal Coos Bay regions. It was shown that the PAM concentrations in those clams living close to industrialized areas were higher than the others living far away from these areas and that, in general, the PAM concentration was higher in spring-summer time than during autumn and winter. The same investigators also discovered [64] that the most hydrosoluble PAHs are those which are concentrated in major proportion in such organisms.

Musial and Uthe [65] described a simple, rapid and easily automatizable method for the determination of PAHs in crustaceans. The method was based on the combination of

chromatographic techniques such as GC and LC for the identification and separation of these compounds and fluorescence spectrometry for quantification. The proposed method was valuable for individual measurements of PAHs in a range of concentration of 0.25-10 ng PAHs/g tissue.

A technique, originally developed to investigate the pollution of Dutch coastal water with metals and PCBs, was modified by Boom [66] for determining the pollution of marine organisms with PAHs. The method was based on the hydrolysis of tissue with 4 M sodium hydroxide, extraction with hexane, clean-up with 10 % deactivated aluminium oxide and quantitative determination with reversed phase HPLC and fluorescence detection. It gave satisfactory results for the analysis of PAHs in mussels.

Trying to determine the bioconcentration factors for petroleum hydrocarbons, PAHs and biogenic hydrocarbons in *Mytilus edulis*, Murray *et al.* [67] applied also this technique. PAHs were quantified by reverse phase HPLC with programmed-wavelength fluorescence detection. The results showed that the bioconcentration factors for PAHs were similar to those found for total hydrocarbons where the major hydrocarbons source was oil, whereas, at other sites, the bioconcentration factors for PAHs were an order of magnitude lower than those determined for petroleum and for hydrocarbons originating from algae.

Perfetti *et al.* [68] proposed modifying the method for determining of PAHs and producing very clean seafood extracts in less than half the time requested previously. After alkaline digestion of seafood, PAHs were partitioned into 1,1,2-trichlorotrifluoroethane. The resulting extract was cleaned up by solid-phase extraction on alumina, silica and C_{18} adsorbents, and then analyzed by gradient reversed phase liquid chromatography with programmable fluorescence detection. Average recoveries of twelve PAHs from five different matrices (mussels, oysters, clams, crabmeat, and salmon) spiked at low ppb levels ranged from 76 to 94 %. The authors obtained results which were in good agreement with the analyses of a mussel standard reference material obtained from the National Institute of Standards and Technology (NIST).

A variant of this methodology (LC/fluorescence) was presented recently by Bouzige *et al.*, [70]. The authors evaluated a new immunoaffinity solid phase extraction (SPE) methodology based on antigen-antibody interactions which was optimized for the selective extraction of PAHs in various complex environmental matrices. This immunosorbent (IS) consists of anti-pyrene antibodies immobilized on a silica support and is used as a classical SPE sorbent. The cross-reactivity of antibodies for analytes structurally related with pyrene allows the simultaneous extraction of the priority PAHs included in the US EPA priority lists. Losses due to the volatility of the two- or three-ring PAHs were avoided by coupling the extraction on-line and using the antipyrene IS with LC. The sensitivity of fluorescence associated with the selectivity of IS allowed the quantification of individual PAHs in contaminated or surface water below the 0.02 µg/l level. Off-line extraction procedures were also set up for the extraction of PAHs from complex solid environmental matrices, such as sludge or mussel extracts. The high selectivity provided by the antibodies permitted the extraction of PAHs and elimination of a great number of interferents in only one step.

Investigating the PAHs pollution in deep-sea environment (1500-1800 m depth), Escartin and Porte [71] developed also a methodology based on the measurement of bile PAHs metabolites in deep-sea fish. The authors selected five species from the NW Mediterranean for the study. Bile crude samples were directly analyzed by HPLC-fluorescence at the excitation/emission wavelengths of benzo(a)pyrene (380/430 nm). The results obtained confirm the long-range transport of PAHs to the deep-sea environment, subsequent exposure of fish inhabiting those remote areas, and fish ability to metabolize and excrete them through the bile.

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