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High-performance liquid chromatographic separation of polycyclic aromatic hydrocarbons using pyridinium chloride as a selective fluorescence quencher to aid detection

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Abstract

The first use of pyridinium chloride (PC), as a selective fluorescence quenching agent of alternant polycyclic aromatic hydrocarbons (PAHs), under HPLC separation conditions is reported. PC was found to be superior to nitromethane, the only reported PAH selective quencher used in HPLC. The mobile phase addition of 0.03 *M* PC greatly simplifies the observed fluorescence-detected chromatograms for complex PAH mixtures, facilitating PAH identification. Stern–Volmer quenching constants (K_{sv}) for PAHs were calculated from the chromatograms obtained under isocratic and gradient conditions and found to be similar. The K_{sv} values were shown to be useful in establishing peak purity.

Keywords: Fluorescence detection; Polynuclear aromatic hydrocarbons; Pyridinium chloride

1. Introduction

The Environmental Protection Agency (EPA) classifies polycyclic aromatic hydrocarbons (PAHs) as priority pollutants. They are pervasive in our environment, and some PAHs are known or suspected carcinogens and/or mutagens. Reversed-phase HPLC is one of the methods commonly used in the separation and/or analysis of PAHs [1–7]. A ''polymeric'' C_{18} column and a mobile phase gradient of water–acetonitrile (ACN) are typically used for such analyses, though other mobile phases such as, micellar modified, have been examined [1–3]. Most PAHs are inherently fluorescent, and utilizing a fluorescence detector in HPLC PAH separations can increase the detection sensitivity to the parts-per-billion level [8,9]. However, fluorescence detection has limitations. For example, it is quite possible to have interfering substances with the same emission wavelength region as the analyte of interest. Even under optimum experimental conditions, it is very difficult, if not impossible, to completely separate all the PAHs present in environmental/biological samples commonly encountered. The spectroscopic interferences can be overcome in part by making the detection selective. For example, setting the excitation and emission wavelengths at their optimum values can result in selective PAH detection [10]. However, real-world samples are usually very complex and their compositions are not known; there-

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fore, it is not practical to use this technique to provide selective PAH detection for such samples.

There are two subclasses of PAHs, alternant and nonalternant. Alternant PAHs (e.g. pyrene) have completely conjugated aromatic systems; while nonalternant PAHs (e.g. fluoranthene) have interrupted aromaticity as a result of five-membered rings in the molecular structure [11,12]. Selective PAH fluorescence-quenching agents can remove the fluorescence signal of one PAH class, without affecting the other class. For example, nitromethane is a known selective photochemical quenching agent [13–34]. It quenches the fluorescence signal of alternant PAHs, leaving that of nonalternants almost unaffected.

It is well known that the extent of quenching might be overestimated or a PAH type might be misidentified, if a quencher shows significant primary or secondary inner-filter effects [35,36]. Unfortunately, nitromethane has a large absorbance in the excitation region of many PAHs. Therefore, the primary inner-filter artifact can become problematic using it in PAH detection. Selective fluorescence quenching was introduced in chromatographic detection in the 1960s [37,38] in thin-layer chromatography, in the late 1970s [16] and early 1980s [10] in liquid chromatography, and in the early 1990s [18] in microcolumn liquid chromatography. Chen et al. systematically investigated the influence of the primary inner-filter effect on the accurate evaluation of fluorescence quenching [18]. In their study, they pointed out that both primary and secondary inner-filter effects could be reduced or even eliminated by using a microcolumn with a capillary flow cell or a modified Stern–Volmer equation with a conventional flow cell. They illustrated that the use of selective quencher facilitated PAH identification and aided in establishing ''peak purity'' [18], a term designed to indicate the presence of ''impurities'' or additional components that coelute with analyte peak and appear as a single chromatographic peak.

Other compounds that do not suffer from inner-filter artifacts have also been identified as selective fluorescence quenching agents for PAHs. For example, cetylpyridinium chloride (CPC), dodecylpyridinium chloride (DDPC), and pyridinium chloride (PC) [39–43] also selectively quench alternant PAHs. Unlike nitromethane, these quenchers are optically transparent in the excitation region of most PAHs, eliminating the primary inner-filter effect. Both CPC and DDPC are cationic surfactants and form micelles with relatively nonpolar interiors that attract hydrophobic PAHs. While they might serve a dual role—as quencher and mobile phase modifier—in the HPLC separation of PAHs, the addition of CPC or DDPC to the mobile phase might also complicate separations. The parent moiety of CPC and DDPC, PC, does not form micelles because of its lack of a nonpolar hydrocarbon chain, making it the preferred option when micelle existence is not desirable. This current study examines the utility of PC, as a selective fluorescence quencher for the separation of complex PAH mixtures, under isocratic acetonitrile and gradient water–acetonitrile HPLC conditions. Our future research will focus on the potential dual role that CPC and DDPC may play in the HPLC separation of PAHs.

2. Materials and methods

A 16-component EPA priority PAH mixture (EPA 16) and the individual PAHs were obtained from AccuStandard (New Haven, CT, USA) at concentrations of approximately 200 ppm in each PAH. The PAH mixture was obtained in a 1:1 methylene chloride–methanol solution. Acenaphthene, acenaphthylene, anthracene, benzo[*b*]fluoranthene, fluorene, indeno[1,2,3-*cd*]pyrene, and naphthalene were obtained in methanol, and phenanthrene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*k*]-fluoranthene, benzo[*a*]pyrene, dibenzo[*a,h*]anthracene, and benzo[*ghi*]perylene were in methylene chloride. Standard Reference Material (SRM) 1597 was purchased from National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA) in toluene. It is a natural, combustion-related mixture isolated from a coal tar sample. Solid PC was obtained from Sigma–Aldrich (St. Louis, MO, USA) and HPLC-grade acetonitrile (ACN) from Fisher (Fair Lawn, NJ, USA). All chemicals were used as received, and HPLC-grade water (resistivity 18 MΩ) was generated in house by an Aqua Solution (Jasper, GA, USA) water system.

Samples were prepared by transferring an appropriate amount of the PAH stock solution into a 2-ml volumetric flask and diluting to volume with 10:90 or 40:60 H_2O–ACN for the isocratic or gradient

experiment, respectively. To prepare the PC-modified mobile phase, solid PC was diluted with an appropriate volume of unmodified mobile phase. Mobile phases were filtered through a 0.45-μm Nylon-66 membrane (Osmonics Laboratory Products, Minnetonka, MN, USA) and degassed under reduced pressure for ~10 min before use.

Chromatograms were collected on a Beckman Coulter System Gold© HPLC (Fullerton, CA, USA), which consists of a dual pump model 126 solvent delivery system, a model 168 photodiode array detector with a 16-μl flow cell, a Jasco FP-1520 fluorescence detector (Tokyo, Japan) with a 16-μl flow cell, and a Reodyne 7725i sample injection valve (Pohnert Park, CA, USA) with a 10-μl sample loop. The two detectors are in sequence. Most experiments were run on a Hypersil PAH column (150×4.6 mm, 5 μm; Keystone Scientific Bellefonte, PA, USA) at room temperature with a 1 ml/min flow-rate. A Vydac 201TP5315 column (150×3.2 mm, 5 μm; Hesperia, CA, USA) was used to test the applicability of the method on columns with different stationary phase preparations. Columns were pre-equilibrated with mobile phase overnight before running the actual isocratic experiments. For the gradient runs, the column was first equilibrated overnight with initial gradient composition, and then re-equilibrated with initial gradient composition until the baseline was flat (~1 h) for subsequent experiments. Under gradient conditions, the concentration of PC quencher was kept the same as in the isocratic experiments—0.03 *M*. Only the EPA 16 sample was examined under gradient conditions, which were as follows: 0–10 min (H_2O–ACN, 40:60), 10–40 min (linearly ramped to 100% ACN), and 40–45 min (100% ACN). The excitation and emission wavelengths for all studies were 320 and 420 nm, respectively. PEAKFIT (AISN Software; Hamilton, New Zealand) was used to deconvolute overlapping peaks, using an exponential modified Gaussian (EMG).

3. Results and discussion

Initial studies, under isocratic conditions, examined the EPA 16 mixture with and without PC (0.01, 0.03 or 0.07 *M*) in the mobile phase, and to test the ''worst case'' scenario where no separation occurs, an alternant and nonalternant PAH were ''forced'' to completely overlap via timed-injection. After each concentration study, the column was re-equilibrated in neat ACN overnight. Chromatograms collected before PC addition and after column re-equilibration were found to be nearly identical, and it was also observed that direct addition of up to 0.07 *M* PC to the mobile phase did not change the PAH separation, allowing the precolumn addition of PC. However, the addition of just 0.03 *M* PC to the ACN mobile phase results in significant simplification of the chromatograms—substantial attenuation of the fluorescence emission signal of alternant PAHs, with little effect on nonalternants. Therefore, 0.03 *M* PC was used for the subsequent studies.

Shown in Figs. 1 and 2 are the fluorescence-detected chromatograms for the EPA 16 and SRM 1597 mixtures from isocratic runs, with and without the addition of 0.03 *M* PC to the mobile phase. After the PC addition, it is obvious that the chromatograms of both mixtures are substantially simplified. The fluorescence emission of alternant PAHs is significantly suppressed, while that of nonalternants is almost unaffected, making nonalternant PAH identification easier. Also note that the time it takes for the separation of both mixtures with ACN mobile phase is less than 15 min, with efficiency of $N \approx 43\,000$ (EPA 16) and 34 000 plates/m (SRM 1597) for benzo[*a*]pyrene, using the Dorsey–Foley equation [44,45]. In order to eliminate the inner-filter effect for nitromethane, Chen et al. used a microcolumn with capillary cell, which resulted in a separation time of 180 min with a methanol mobile phase [18]. While a different mobile phase would lead to different retention indices, the use of a microcolumn is expected to be primarily responsible for the long elution time in their study. In our study, there is no inner-filter correction necessary because PC is optically transparent in the excitation region utilized. Nevertheless, as a control, the eluate absorbance was monitored with the photodiode array detection system.

The quenching mechanism of PC is known to be dynamic [42]; therefore, the Stern–Volmer equation was used to calculate the quenching constants (K_{sv}) [46]:

$$F_0/F - 1 = K_{sv}\,[\text{Quencher}]$$

In this equation, F_0 and F are the integrated areas

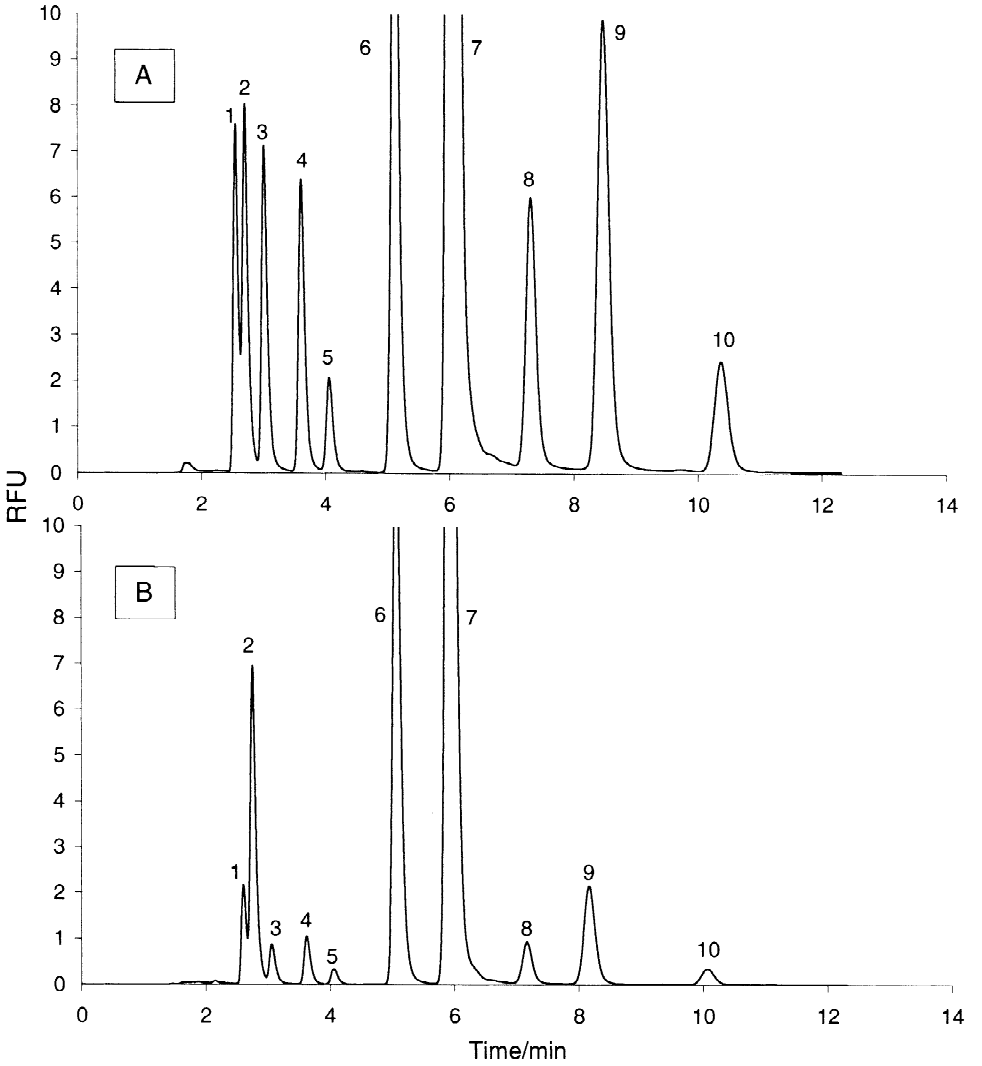

Fig. 1. Chromatograms of EPA 16 in neat ACN (A) and ACN with 0.03 *M* PC (B). Detected component identification: 1=anthracene, 2=fluoranthene, 3=pyrene, 4=benzo[*a*]anthracene, 5=chrysene, 6=benzo[*b*]fluoranthene, 7=benzo[*k*]fluoranthene, 8=benzo[*a*]pyrene, 9=dibenzo[*a,h*]anthracene and 10=benzo[*ghi*]perylene.

under the fluorescence-detected PAH peaks in absence and presence of quencher, respectively; K_{sv} is the Stern–Volmer quenching constant and [Quencher] is the quencher concentration. Since the column is pre-equilibrated, it is assumed that the postcolumn quencher concentration is the same as the ''bulk'' mobile phase. Note that in Figs. 1 and 2 the quenching of alternant PAHs is only partial. According to the Stern–Volmer equation, the quenching is proportional to the concentration of quencher in solution. Therefore, if complete quenching is desirable, more quencher could be added to the mobile phase.

It is difficult to get baseline separation for all the PAHs present in complex PAH mixtures with reasonable retention times; therefore, to obtain F_0 and F for overlapping PAHs, the software PEAKFIT was used to deconvolute them. However, how well the deconvolution represents the experimental data strongly depends on the extent of peak overlap. This is a

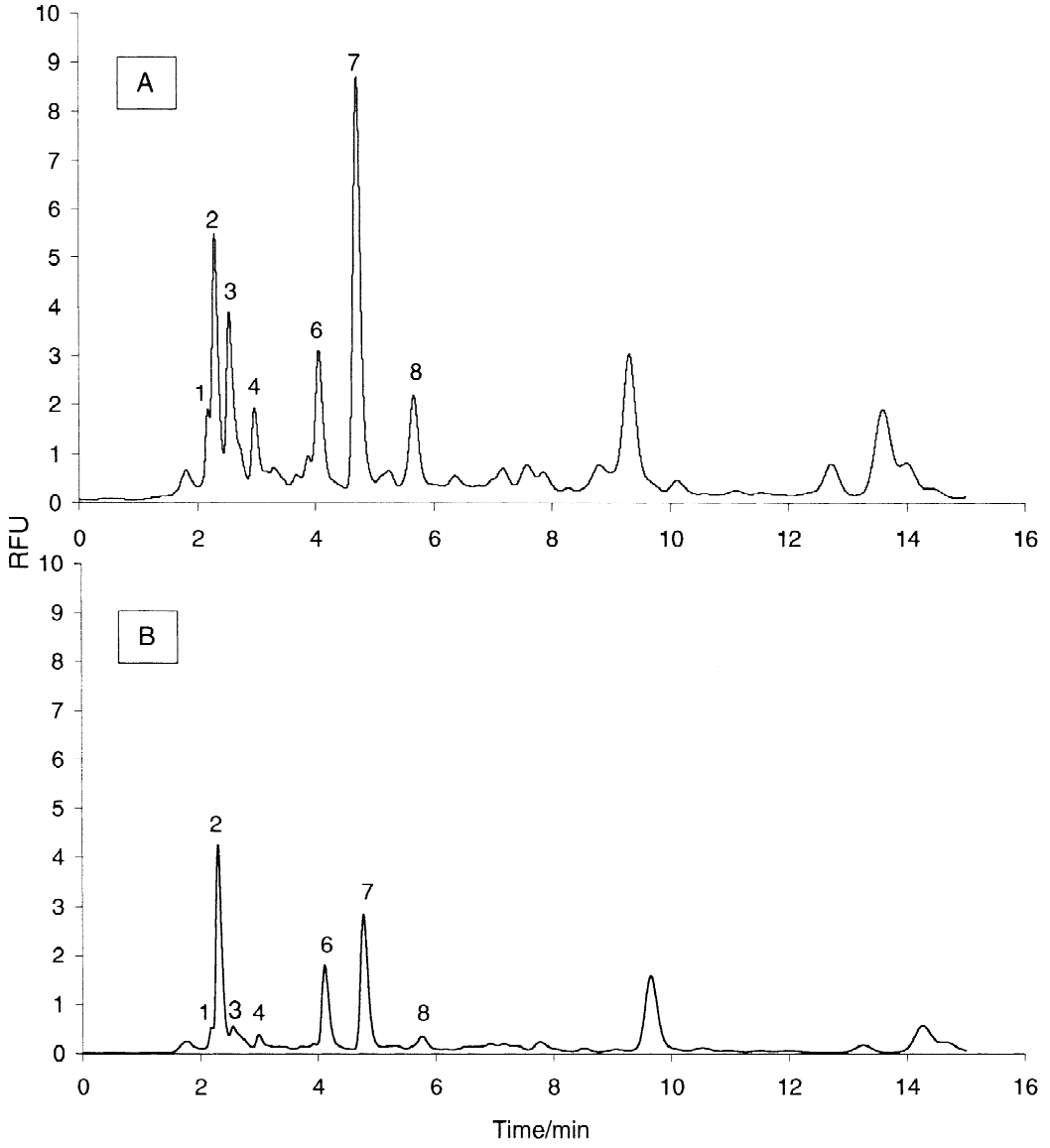

Fig. 2. Chromatograms of SRM 1597 in neat ACN (A) and ACN with 0.03 *M* PC (B). The PAH identification numbers are as in Fig. 1.

mathematical issue—the greater extent of peak overlap, the larger number of possible fitting models and the larger the deviation. Therefore, by staggering the injection time of two individual PAHs (one alternant and one nonalternant), we examined how the fitting was affected by the extent of peak overlap. The resulting chromatographic peaks were deconvoluted with a two-component fit, representing the actual number of components. It was found that some chromatographic resolution ($R \approx 0.35$) was needed in the experimental data for the deconvolution to be representative. Deviation from the actual data was large if the two peaks totally overlapped.

The Stern–Volmer quenching constants (K_{sv}) were calculated for several PAHs from the chromatograms in Figs. 1 and 2. The results, summarized in Table 1, did not need to be corrected for inner-filter effects. For comparison, quenching constants reported from

Table 1
Stern–Volmer quenching constants (K_{sv}) for alternant and nonalternant PAHs

PAHs	Peak no.[a]	K_{sv} (M^{-1})			
		Bulk[b] Literature	Isocratic SRM 1597	Isocratic EPA 16	Gradient EPA 16
Alternant PAHs					
Anthracene	1		121±10	85±4	46±3
Pyrene	3	1896±48	363±52	235±10	233±8
Benzo[*a*]anthracene	4		173±19	177±10	134±6
Chrysene	5			177±14	142±5
Benzo[*a*]pyrene	8		219±12	174±8	153±26
Dibenzo[*a,h*]anthracene	9			128±3	132±37
Benzo[*ghi*]perylene	10			204±5	177±23
Nonalternant PAHs					
Fluoranthene	2	2±1	11±1	4±1	1±1
Benzo[*b*]fluoranthene[c]	6	16±2	26±1	5±1	17±4
Benzo[*k*]fluoranthene[c]	7	2±1	62±2	49±2	

[a] Shown in Figs. 1 and 2.
[b] [PC] = 0.01 *M* for all PAHs except pyrene (0.006 *M*)—there was a typographical error in the original reference [42].
[c] Diluted sample for HPLC analysis.

bulk solution studies [42] are also listed. Comparisons can be made because the K_{sv}, the slope of a Stern–Volmer plot, is concentration independent within its linear range. The first three-analyte peaks of EPA 16 and all the identifiable peaks of SRM 1597 were deconvoluted. Since the number of components is known and information about the individual components was previously obtained, deconvolution of the EPA 16 chromatograms was achieved by allowing a fixed number of peaks to be assigned to each convoluted peak. However, for the highly complex SRM 1597 sample, it is not possible to know exactly how many components are under each peak; therefore, using the literature [18] and SRM 1597 mixture certificate of analysis provided by NIST as a guide, a reasonable number of peaks were assigned to each complex peak. The number of peaks assigned for each peak was the same for all replicates. The goodness of fit coefficients (r^2) for all deconvolutions were better than 0.999.

Examination of the data in Table 1 indicates that the quenching constants (K_{sv}), obtained under isocratic conditions for the PAHs in the two mixtures, are similar. For example, the K_{sv} values for benzo[*a*]pyrene are large in both mixtures, illustrating that PC quenches this alternant PAH. Conversely, the K_{sv} values for nonalternant fluoranthene are relatively small. The anomalous behavior of benzo[*k*]-fluoranthene, illustrated by its unexpectedly high K_{sv} value, has been noted in several other systems [35,36]. This is a quantitative illustration of the selective quenching seen in Figs. 1 and 2. The K_{sv} values may also aid in the qualitative identification of PAHs, assuming well-controlled separation conditions (i.e. temperature, pressure, injection volume, etc.). Further examination of Table 1 shows that quenching constants obtained from SRM 1597 are always larger than those from EPA 16. This trend is not surprising, considering the complexity of the SRM 1597, and was also seen in studies utilizing nitromethane [18]. As mentioned earlier, the coal tar extract includes many unknowns; therefore, it is possible that traces of unknown alternant PAHs coelute with the known PAHs and give slightly larger K_{sv} values. In addition, note that the quenching constants from bulk solution and chromatographic studies for the PAHs are also similar, with the exception of pyrene, which has been noted previously [18,39–43]. Some differences in the K_{sv} values between these two types of studies are expected. The excitation wavelength and emission wavelength(s) are different. Also, the bulk solution studies employed single point intensity determinations versus an integrated area determination for the HPLC data, as suggested by Chen et al. [18]. Given these facts and the nature of the ‘‘assumptions’’ used in the

fitting of the complex chromatographic data, this comparison illustrates the robustness of the K_{sv} values and further supports their use in facilitating PAH identification.

The calculated Stern–Volmer quenching constants for the PAHs that were forced to totally overlap are listed in Table 2. These quenching constants were calculated without deconvoluting the data. Entries in Tables 1 and 2 show that the quenching constants obtained for an alternant and nonalternant PAHs that completely overlap are between the individual PAH values, as predicted by the Stern–Volmer equation. One also expects that two overlapping PAHs of the same type would result in a larger K_{sv} than what is predicted for either individual component alone. Therefore, the K_{sv} values can be used to establish peak purity, indicating when coelution occurs. It is especially important to make this determination prior to any quantitative analysis.

As mentioned in the Introduction, a water–ACN gradient is commonly used in the PAH separations. Therefore, it is essential to examine the compatibility of the PC selective quenching method under gradient conditions. Chromatograms of EPA 16 were collected with and without the addition of 0.03 *M* PC to the gradient mobile phase and then analyzed. The K_{sv} values of several PAHs were compared to those obtained under isocratic ACN conditions (Table 1). These values represent the quenching efficiency of PC under isocratic and gradient conditions and are similar. As under isocratic conditions, quenching constants are large for alternant PAHs and small for nonalternant PAHs under gradient conditions. Since the mobile phase composition changes under gradient conditions, from 40:60 ACN–H_2O to 100% ACN, it is apparent that the PC quenching selectivity is retained at these ACN and H_2O percentages. Therefore, this method is also applicable under this gradient condition; however, solvent polarity is known to affect nitromethane selective quenching [23,24]. In our case, the mobile phase composition, across the gradient, remains very polar. If the polarity of mobile phase is considerably altered (e.g. 40:60 toluene–acetonitrile and ethyl acetate–acetonitrile [23,24]), the extent of selective quenching will also be changed.

Table 2
Stern–Volmer quenching constants (K_{sv}) of PAHs that completely coelute via timed-injection, under isocratic conditions

PAH mixture	K_{sv} (M^{-1})
Pyrene–fluoranthene	41±3
Benzo[*a*]anthracene–benzo[*b*]fluoranthene	16±1
Anthracene–fluoranthene	41±2

PC's selective fluorescence quenching of alternant PAHs under HPLC conditions was also tested in the same manner on a Vydac 201TP5315 column, which is also used for PAH separations. It was found that the selective quenching is column independent, though some separation parameters, such as retention time and resolution, vary from column to column. The Vydac 201TP5315 column differs from the Keystone PAH column in terms of stationary phase substrate, carbon loading, alkyl chain conformation, end-capping, etc. Readers are reminded that quenching selectivity, not chromatographic selectivity, is retained.

The goal of the PC selective quenching method is for it to be used in the separation and characterization of real-world, PAH-containing samples. Such samples are usually much more complex than standards such as EPA 16. For example, in the EPA 16 sample, the PAHs are all the same concentration, 200 ppm. However, while the SRM 1597 sample is a standard reference material, it is also a natural, combustion-related PAH mixture isolated from a coal tar sample with a range of PAH concentrations. In fact this sample is more complex, specifically in terms of the number of PAHs and range of PAH concentrations, than many real world samples found in the literature such as, sediment, sea water, soil and foods [4,6,7]. Not surprisingly, its complexity more closely resembles other coal-derived real world samples such, as waste oils [47]. Therefore, the inclusion of the SRM 1597 sample illustrates the utility of the method for even complex environmental samples.

Polycyclic aromatic hydrocarbons are just one class of polycyclic aromatic compounds (PACs) that might be present in real-world samples. PAH derivatives and other PACs such as, polycyclic aromatic nitrogen/oxygen/sulfur heterocycles (PANHs, PAOHs, and PASHs, respectively) and their derivatives, also exist, and these compounds respond differently to the presence of selective quenching

agents [25,27,28,48,49]. For example, there are more exceptions to the selective quenching rule with PANHs, and PAOHs/PASHs respond to the selective quenchers as substituted PAHs because the heteroatom disrupts the aromaticity [11,25, 27,28,48,49]. To further examine how PC selective quenching is affected by substitution, we studied two substituted PAHs, 1,5-dimethylpyrene and 10-methylbenzo[*b*]fluoranthene. Under isocratic conditions, the K_{sv} values of 1,5-dimethylpyrene (224±8) and pyrene (235±10) or 10-methylbenzo[*b*]fluoranthene (14±1) and benzo[*b*]fluoranthene (5±1) are similar. Therefore, as with any complex sample, use of selective fluorescence quenching facilitates PAH identification in conjugation with other information from the separation such as, the retention index.

4. Conclusions

Pyridinium chloride, the selective PAH florescence-quenching agent, was introduced into the HPLC separation of complex PAH mixtures without the need for inner-filter correction. Chromatograms of two samples (EPA 16 and SRM 1597) that span a wide range of complexity were substantially simplified after the addition of 0.03 *M* PC to the mobile phases. Stern–Volmer quenching constants (K_{sv}) were obtained from the chromatograms for several PAHs and found to be in good agreement with each other and with bulk solution studies. Therefore, K_{sv} values are useful in facilitating PAH identification in complex samples. The method was shown to be applicable under the isocratic ACN and gradient H_2O–ACN conditions. Quenching constants were also calculated for PAHs forced to totally overlap, by treating them as a single peak. The results indicate the possible use of K_{sv} in the establishment of peak purity for an apparent single chromatographic peak. This study clearly demonstrates the effectiveness and superiority of PC over nitromethane as a selective fluorescence quencher for HPLC PAH separations. The judicious use of selective quenching agents facilitates PAH identification and establishment of peak purity, aiding in the analysis of complex environmental/biological samples.

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