Experimental Artifacts and Determination of Accurate Py Values

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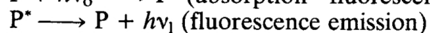
Recently the Py solvent scale has been introduced and a large body of data has been generated using fluorescence measurements. Numerous problems exist in the correct determination of these values, including instrumental and chemical artifacts. Among the instrumental problems associated with correct Py assignments are slit width effects, inner filtering and efficiencies associated with double-pass *vs.* single-pass cell compartment designs. These instrumental problems, together with chemical artifacts related to adequate blank correction and temperature control, were investigated in the work reported in this paper.

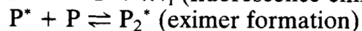
Keywords: *Py solvent polarity scale; instrumental artifacts*

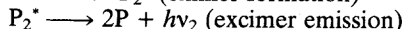
Empirical solvent polarity parameters represent simple and straightforward means of characterising solvent media. Among the more well known parameters are the Hildebrand solubility parameter,[1] the Kosower Z parameter,[2] the Dimroth E_T parameter,[3,4] the Taft π^* parameter[5,6] and the Py scale.[7,8] Factor analysis and multiple regression show that the various parameters contain different information regarding solvent structure. For aprotic solvents, E_T is a measure of polarity, polarisability and cohesion to the extent of 43, 39 and 18%, respectively. In comparison, these effects are weighted differently, *i.e.*, 53, 18 and 29%, for π^*.[9]

Our interest in solvent polarity concerns the experimental determination of Py values as this scale has been shown to facilitate mechanistic understanding of solute retention in reversed-phase liquid chromatography.[10–12] Pertinent photophysical properties of pyrene in fluid solutions are

$P + h\nu_0 \longrightarrow P^*$ (absorption - fluorescence excitation)


$P^* \longrightarrow P + h\nu_1$ (fluorescence emission)


$P^* + P \rightleftharpoons P_2^*$ (eximer formation)


$P_2^* \longrightarrow 2P + h\nu_2$ (excimer emission)

The emission spectrum of the monomer consists of five major vibronic bands labelled I–V in progressive order, *i.e.*, the $0 - 0$ band being labelled I, etc. The intensities of various bands show a strong dependence on the solvent environment. A significant enhancement is observed in the $0 - 0$ vibronic band intensity in the presence of polar solvents. The ratio of emission intensities for bands III and I (Py = I/III) serves as a quantitative measure of solvent polarity and structure.

Solvent classification based on Py values requires that the experimentally determined ratio of emission intensities be free of instrumental artifacts that may lead to discrepancies in the literature. In this paper we discuss the instrumental artifacts and chemical problems that lead to irreproducible Py values. Among the instrumental problems that we have encountered are inner filtering, finite slit effects, the efficiency of the double-pass cell configuration and the manipulation errors arising with computer acquisition and massaging of data. One chemical artifact that is also apparent is the effect of temperature on the efficiency of fluorescence emission.[13]

Experimental

Pyrene (Aldrich, >99%) was recrystallised three times from methanol and dried at 80 °C prior to use and a stock solution was prepared in chloroform (5.7×10^{-4} M). Small aliquots of the stock solution were transferred into test-tubes, allowed to evaporate and then diluted to 5 ml with the solvent of interest. The final pyrene concentration was 1×10^{-6} M except where stated otherwise. All solvents were of HPLC, spectroquality or analytical-reagent grade, purchased commercially from Aldrich or Fisher Scientific, and the resulting solutions were optically dilute (absorbance $cm^{-1} < 0.01$ at all wavelengths investigated) except where stated otherwise.

Absorption spectra were recorded on a Cary Model 118 C spectrophotometer in the usual manner using 1-cm quartz cells. The fluorescence experiments were run on a Perkin-Elmer MPF-44B or LS-5 spectrofluorimeter with a Model 3600 data station in a 1-cm square cuvette. All data were accumulated at 21 °C. Spectra obtained on the LS-5 were usually averages of five scans, which were then blank corrected except where the blank corrections were investigated. The single- *vs.* double-pass emission experiments were similar to those performed for excitation inner filtering by Street and are adequately described elsewhere.[14,15] Briefly, the emission inner filtering experiments were performed for a "single-pass configuration" by inserting a piece of black paper between the cuvette and the emission mirror scribed into the cell holder of the LS-5 and then recording the spectra.

Results and Discussion

Inner filtering is a major problem associated with obtaining correct fluorescence data, which assumes that the sample is optically dilute (A $cm^{-1} < 0.01$) at all analytical wavelengths. In emission experiments (as in Py determination) primary inner filtering (*i.e.*, the absorption of large amounts of excitation radiation) can be ignored as the excitation wavelength remains constant and consequently the absolute values obtained from the emission experiment are independent of small amounts of this type of inner filtering. This assumption may not be true where large amounts of inner filtering are involved in conjunction with large cuvettes, high absorbances and in the recording of high resolution spectra, as is required in the Py determination.

For secondary inner filtering (*i.e.*, the absorption of large amounts of fluorescence emission) there are two areas of concern, the first and most predominant of which is the preferential absorption of the 373 nm emission by the sample. This effect is clearly demonstrated in Fig. 1 and in Table 1, which summarises data from single- and double-pass experiments for the determination of Py in methanol. (Note: our Perkin-Elmer LS-5 fluorimeter records peak I at 373 nm although the Cary instrument confirms the location of this peak at 371 nm; small discrepancies such as this are unavoidable.) The absorbance of all solutions tested in the 1×10^{-3}–2×10^{-4} M concentration range exhibits inner filtering of the 373 nm peak (peak I) whereas the solutions are transparent at the 383 nm peak (peak III), leading to significantly lower values for the intensity of the 373 nm emission. As Py is calculated as the intensity of band I divided by the intensity of band III, all solutions having concentrations of pyrene > *ca.* 10^{-4} M will exhibit lower than expected Py values for

Table 1. Effect of inner filtering on Py determinations in methanol

Pyrene concentration/M	Absorbance cm^{-1}		Py	
	371 nm	383 nm	Single-pass	Double-pass
9.4×10^{-4}	0.504	0.030	0.643	0.624
7.3×10^{-4}	0.450	0.024	0.769	0.718
5.6×10^{-4}	0.325	0.018	0.911	0.815
3.8×10^{-4}	0.227	0.012	1.065	0.974
1.9×10^{-4}	0.095	0.006	1.156	1.104
1.9×10^{-6}	$<10^{-3}$	$<10^{-3}$	1.221	1.208

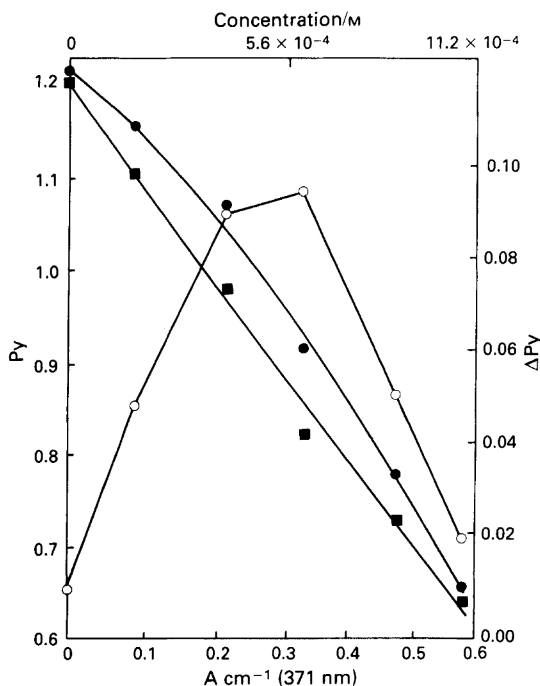


Fig. 1. Inner filter effect on determination of Py for methanol. Data taken on LS-5 spectrofluorimeter using 3-nm emission slit. ●, Single-pass experiment; ■, double-pass experiment; ○, ΔPy calculated as (single-pass Py − double-pass Py)

methanol. Further, the absorption of light is much greater in the 300–340 nm region for pyrene, indicating that for complete removal of primary inner filtering, the concentration of pyrene used in the determination of Py values for methanol should be less than 3.8×10^{-6} M. The concentrations employed will differ from one solvent system to the next as the molar absorptivities at all wavelengths involved are solvent dependent. From the data at a pyrene concentration of 2×10^{-4} M it is obvious that even small amounts of secondary inner filtering will have a pronounced effect on the experimentally determined value of Py. A further consequence is that instruments that are designed to enhance a signal by placing mirrors about the sample cuvette leading to a "double-pass" configuration will exhibit a greater inner filter effect. It is interesting to compare the plots of Py *vs.* absorbance at 371 nm for the single- *vs.* double-pass configurations that converge on the same Py values for either optically dilute or dense solutions (Fig. 1). This is a function of the fact that the total emission intensity, F_T, is composed of contributions from the single-pass experiment, F_1, and the second pass, F_2, of the double-pass configuration according to

$$F_T = F_1 + F_2 \quad \quad (1)$$

From the simulated plots of F_1 and F_2 in reference 14, $F_2 \rightarrow 0$ as Abs $\rightarrow \infty$. Therefore, at high absorbance values,

$$F_T = F_1 \quad \quad (2)$$

Equation 2 is also the exact relationship for the single-pass fluorescence experiment. These plots do not converge on exactly the same value because the reflectivity terms, R_m, derived in reference 14 and evaluated in reference 15 are not identical at 373 and 383 nm, favouring the intensity of III at 383 nm. This artifact leads to slightly higher Py values for the single-pass experiment at all values, especially under optically dilute conditions. In the preceding we have only treated self inner filtering, *i.e.*, the attenuation of signals owing to absorption by the fluorophore itself; however, the solvent may contribute to this effect as well. For example, isobutyl methyl ketone is reported to have a Py value of 1.48 and absorbances of 0.06 and 0.02 at 370 and 380 nm, respectively.[16] Whereas this would only contribute little more than experimental error to the determination of Py in the single-pass experiment, it would contribute a significant error to the double-pass experiment. Further, this solvent and numerous others show such high light absorption below 350 nm that the conventional fluorescence determination of Py is extremely questionable because the primary inner filtering effect has never been evaluated.

The second inner filtering situation arises from sample turbidity, which gives rise to a situation similar to "absorbance" at all wavelengths. Although the absorbance will be wavelength dependent, we can assume that it will be fairly constant over the small wavelength range associated with the fluorescence Py determination, leading to a more significant attenuation of F_2 at all wavelengths. As the inner filtering attenuation is now constant for peaks I and III, the only effect that should be introduced by sample turbidity should be a slight increase in the Py value associated with the removal of the R_m effect incorporated into F_2, as noted in Fig. 1 and discussed previously. We have recently encountered solvent turbidity problems in the determination of Py for binary solvent systems commonly employed as mobile phases in HPLC. The turbidity may be a result of the way in which the solvents were prepared by the manufacturer. Some other researchers have made this same assumption for the mechanistic evaluation of chromatographic stationary phases although the validity of the assumption has never been evaluated.

The effect of slit width on the determination of Py is summarised in Table 2 and Fig. 2. A general rule in spectroscopy is that for accurate spectra, the spectral slit width should be not more than one tenth of the natural band width of the chromophore or fluorophore under investigation. Using the UV spectra we have estimated the peaks in the pyrene spectrum to have full widths at half-maximum of the order of 3 nm in the 380 nm region. This implies that for accurate spectra the maximum spectral slit width allowable for the correct determination of Py should be less than 0.6 nm, which is five times smaller than the smallest emission slit width setting on the LS-5 spectrofluorimeter. In practice we have found that 1.0 nm slits are sufficiently narrow to obtain correct Py values and that narrower slits only degrade signal to noise ratios, which increases the imprecision in Py values (Fig. 2). Our values for Py determination on the LS-5 are consistently 10% lower than those reported by Dong and Winnik,[8] which are a direct result of degraded resolution of the pyrene spectra

Table 2. Effect of emission slit width and excitation wavelength on determinations of Py

Solvent	Reported* value	Py value: MPF-44B 308 nm	MPF-44B 338 nm	LS-5 308 nm	LS-5 338 nm
Cyclohexane	0.58	0.658(13.4)†	0.568(−2.1)†	0.580(0.0)†	0.544(−6.2)†
Butanol	1.06	0.947(−10.7)‡	1.061(0.0)	0.862(−18.7)	0.862(−18.7)
Chloroform	1.25	1.395(12.0)		1.177(−5.8)	1.161(−7.1)
		1.356(8.5)	1.364(9.1)	1.201(−4.0)	1.190(−4.8)
					1.195(−4.4)§
Methanol	1.35	1.477(9.6)		1.23(−8.8)	
		1.443(6.9)	1.38(2.2)		
Tetrahydrofuran	1.35			1.22(−9.6)	
Acetonitrile	1.79	1.930(7.8)		1.59(−11.2)	
Water	1.87	1.973(5.3)		1.62(−13.4)	
Dimethyl sulphoxide	1.95	2.186(12.3)		1.77(−9.2)	
Average deviation		+9.5 ± 2.9¶		−9.0 ± 5.4¶	

* Reported in reference 8.
† Percentage deviation from value reported in reference 8.
‡ Omitted from calculation of deviation. Data used to determine Py had a low signal to noise ratio.
§ Average of 25 scans used for Py calculation. All other data reported for LS-5 are averages of five scans.
¶ Calculated as σ_{n-1}.

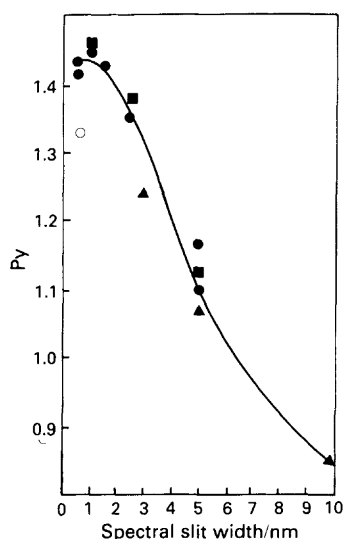


Fig. 2. Effect of emission slit width on determination of Py for methanol. ●, Using an MPF-44B spectrofluorimeter with a 20-nm excitation slit; ■, using an MPF-44B spectrofluorimeter with a 10-nm excitation slit; ▲, using an LS-5 spectrofluorimeter with a 10-nm excitation slit. All data at an excitation wavelength of 308 nm and 1×10^{-6} M pyrene. ○, Indicates low signal to noise data. See text for explanation

Table 3. Effect of computer acquisition and manipulation of data on determination of Py in chloroform

Pye value: Blank corrected	Without blank correction	Comment
For data presented in Fig. 4—		
1.287	1.249	Single scan—no data massage (Peak III difficult to identify)
1.185	1.144	Average of 5 scans
1.303	1.236	Single scan smoothed by a 9-point function
Data similar to Fig. 4 using 1×10^{-6} M *pyrene—*		
1.201	1.212	Single scan—no data massage (Peak III difficult to identify)
1.201	1.190	Average of 5 scans
1.205	1.200	Single scan smoothed by a 9-point function
Average of 25 scans on 1×10^{-6} M *pyrene—*		
1.196		—
1.199		Smoothed by a 5-point function
1.196		Smoothed by a 9-point function
1.184		Smoothed by a 13-point function
1.133		Smoothed by a 19-point function, peak II at 380 nm is lost

in conjunction with small solvent induced wavelength shifts (typically 1 nm) of adjacent bands into and out of the peaks used in the determination of Py. On the other hand, the Py values obtained from the MPF-44B are consistently 10% higher than those reported by Dong and Winnik[8] even though these authors reportedly used a 0.5 nm slit width *vs.* the 1.0 nm slit width used in obtaining the data with the MPF-44B. Other effects such as detector response *vs.* wavelength may have caused these deviations in their Py values, which we assume to be correct.

Although blank corrections are negligible for most solvents, in a few instances the corrections were greater than the experimental error. The error is minimised by using 10^{-6} M pyrene, the highest concentration permissible in order to avoid primary and secondary inner filtering. For instruments that discriminate against primary inner filtering (*e.g.*, front surface sample illumination) pyrene concentrations of up to 1×10^{-4} M are acceptable. At lower pyrene concentrations and higher amplifications several blanks were not constant or negligible *vs.* wavelength. Most of the deviations that we have observed increase with increasing wavelength for excitation at 308 nm. In other words the correction is higher for peak III and the uncorrected value is always higher than it should be. As pyrene absorbs more strongly in the 340 nm region, primary inner filtering occurs at 1×10^{-6} M concentrations. We have not observed significant differences between Py values obtained at 308 nm excitation (negligible primary inner filtering) and excitation at 338 nm (moderate primary inner filtering) for double pass configuration. The blanks, however, for very dilute pyrene concentrations (1×10^{-7} M), where all inner filtering is completely eliminated, contain Raman bands of the solvent. For example, the Raman band for cyclohexane is at 375 nm and severely overlaps the analytical wavelength for Py determination making manual blank correction difficult (Fig. 3). We have noted that with low signal to noise ratio data, we tend to manually undercorrect for the blank leading

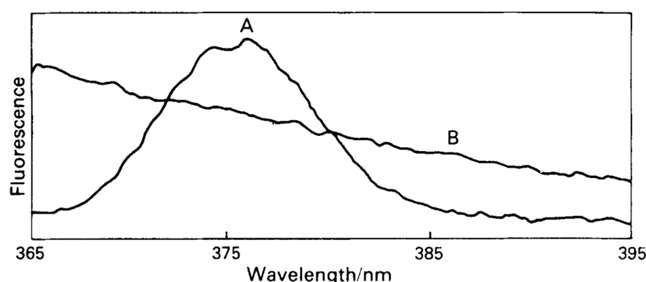


Fig. 3. Emission spectra for cyclohexane blanks accompanying Py determination for 1 × 10^{-7} M pyrene. Data taken on LS-5 spectrofluorimeter with both slits 5 nm to enhance Raman band in A. A, Excitation at 338 nm; and B, excitation at 308 nm

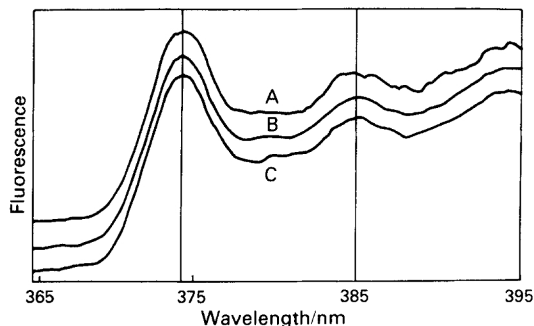


Fig. 4. Effect of computerised data acquisition on Py determination for chloroform. Data taken on LS-5 spectrofluorimeter with an excitation wavelength of 308 nm and excitation and emission slits of 10 and 3 nm. The sample is 1 × 10^{-7} M pyrene. A, Single scan offset by 10 scale divisions; B, average of 5 scans offset by 5 scale divisions; and C, accumulation of 5 scans zeroed at 365 nm

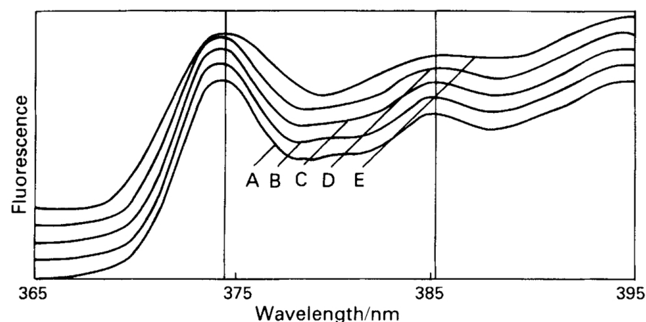


Fig. 5. Effect of applying smoothing functions on Py determination for chloroform. A, Blank corrected average of 25 scans on 1 × 10^{-6} M pyrene sample (see Fig. 4 for instrument settings); B, smoothed by 5-point function and offset 5 scale divisions; C, smoothed by 9-point function and offset by 10 scale divisions; D, smoothed by 13-point function and offset by 15 scale divisions; and E, smoothed by 19-point function and offset by 20 scale divisions

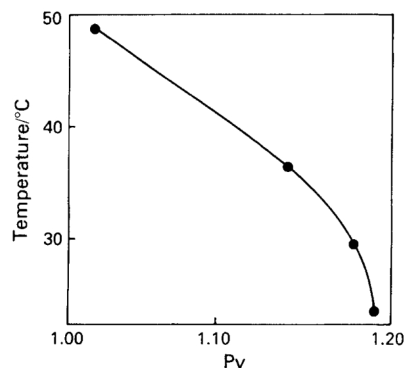


Fig. 6. Apparent effect of temperature on Py determination for butyl acetate. See Fig. 4 for instrument and sample conditions

to low Py values. This may account for the trend of lower than expected Py values at slit widths less than 1.0 nm seen in Fig. 2. This perhaps accounts for the deviation between our data and that of Dong and Winnik[8] summarised in Table 2.

For the dilute solutions of pyrene used, the spectra are often noisy and as a direct result computer manipulation and acquisition of data are extremely attractive. In addition to spectral subtraction (*i.e.*, blank correction) we have used spectral averaging, spectral accumulation and smoothing of single spectra. Fig. 4 shows the emission scans for 1 × 10^{-7} M pyrene in chloroform solution. The accumulated and averaged spectra are of comparable quality although the single scan is ambiguous as to the exact location of peak III. The five scans take considerably longer than the application of software smoothing of single spectra. Fig. 5 shows the effect of smoothing excellent data (25 scans averaged). Table 3 summarises the Py values obtained from the corresponding smoothing. It is apparent from Fig. 5 that spectral distortions occur using relatively small smoothing functions, even on low resolution data. This leads to significant errors in Py determinations, as evidenced by the loss of peak II at 380 nm when using higher smoothing functions. It is expected that the smoothing of higher resolution data would cause large errors even for smaller smoothing functions. Single slow scans using very long pen responses can contribute obvious errors; however, when applied correctly, these yield accurate Py values.

In the early stages of work with binary solvent systems,[12] we experienced large deviations between Py values for rapid, successive scans of the same samples. We later attributed this trend to a temperature effect brought about by the heat of mixing of the solvents, which cooled during a spectral scan of the sample owing to rapid heat transfer from the cuvette to the massive sample block. In a later experiment a pyrene - butyl acetate sample was heated to about 70 °C and allowed to cool in the instrument while scans were taken. Fig. 6 shows the apparent effect of temperature on the experimentally determined value of Py. The correct interpretation of this data was determined from values taken during a thermostated experiment in which the cell block was preheated. At elevated temperatures the fluorescence spectrum of pyrene is less intense. The sample cools by several degrees during the 30 s required to acquire the data between the I and III peaks, which allows the III peak intensity to build up relative to the I data that has already been stored. The temperature drop caused by heat transfer to the sample block during the interval required for data accumulation is greater at higher temperatures, leading to lower apparent Py values at higher temperatures. In the "thermostated" experiment the data were taken more rapidly and the effect was greatly diminished, although the same trend persisted. Unfortunately, we do not have a thermostated cell in which to test this hypothesis fully, but this problem should be borne in mind when appreciable heats of mixing are involved or when techniques such as oven drying of cuvettes are employed, etc. We are continuing work on this problem as it may contribute to the deviation between our high resolution data and that of Dong and Winnik[8] (Table 2). An earlier study by Hara and Ware[13] suggested that the Py values are not only temperature dependent but that the variations are more pronounced for polar solvents.

Conclusion

The Py scale has been demonstrated as an adequate probe of solvation ability. This system is very close to ideal because fluorescence is inherently more sensitive than absorption experiments and because pyrene is sufficiently soluble in all solvents for an adequate determination of Py to be carried out. However, numerous effects may be encountered that contribute to the deviation between Py values from different

laboratories. We recommend that a standard set of conditions be adopted in the subsequent reporting of Py values. These conditions should be based on our observations and should include the following specifications. 1, A standard 10^{-6} M pyrene solution should be employed for all Py determinations. 2, The narrowest of emission slit settings should be employed with 1.0 nm as the recommended minimum value. 3, As many instruments do not have this high a resolution, several standard solvents spanning the Py range should be employed as reference calibrants. We recommend cyclohexane, chloroform, methanol and water, which are commonly available in high quality. 4, The effect of temperature on the value of Py is still speculative and hence it is recommended that the temperature at which the data was taken be reported. It is hoped that the use of calibration standards may help to minimise any impact on Py values that may result from determinations at different temperatures.

The authors thank Karl Pearson and the Cleveland State University for the use of the Perkin-Elmer MPF-44B spectrofluorimeter. Acknowledgement is made to the Donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research.

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Paper A6/38
Received February 10th, 1986
Accepted May 19th, 1986