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Importance of Spectral Correction in Fluorescence Spectroscopic Studies of Crude oils and Asphaltenes

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ABSTRACT

Fluorescence spectroscopy has been widely used in biochemistry to elucidate molecular interactions. Likewise, it has also been applied to petroleum systems and in particular asphaltene association by concentration effects. It is also a common method for on-line measurements of oil-in-water in offshore oil and gas industry. Due to the complexity of crude oil and asphaltenes many assumptions and corrections are necessary in an adequate analysis especially of concentration effects. Unfortunately, many studies in the literature lack appropriate incorporations of simple effects such as re-absorption effects (inner filter) and energy transfer (FRET, fluorescence resonance energy transfer) among molecules leading to apparent red shifts. The latter has wrongly been reported as an effect of molecular association. In the present paper, we analyze the "Petrophase 2017" asphaltenes and crude oil using

emission spectra (λ_{ex} 300 and 400 nm) and synchronous spectra as a function of concentrations in toluene between 0.2 and 1000 ppm in both Front Face (FF) and Right Angle (RA) irradiation geometries. Inner filter effects are seen in both configurations leading to complete loss of fluorescence at short wavelength. After corrections, we observe that linearity in intensity-concentration relations is almost restored, but some redshifts remain. This is mainly due to FRET and hence not related to true molecular aggregation. We further explore a number of Stern-Volmer based approaches to elucidate mechanisms of molecular interaction using fluorescence spectroscopy. The SV plots indicate that fluorophores could be shielded at quite high concentrations in agreement with asphaltene aggregation. The presence of FRET indicates that some molecules contain several covalently bonded but independent fluorophores. The conclusion is that caution is needed in interpretation of concentration effects in fluorescence spectroscopy studies of petroleum fractions and all possible corrections should be made before concluding on molecular interactions such as critical nano-aggregate concentration phenomena.

INTRODUCTION

Fluorescence spectroscopy is widely used as a sensitive technique to analyze and understand molecular interactions in solution of fluorophores particular within biochemistry. [1-5]However, due to the sensitivity of the technique artefacts may arise in the recorded spectrum due to both concentration and optical effects. Different excitation-emission configurations can be used in fluorescence studies. Most common are the right-angle (or 90^{o}) configuration which is a transmission mode with constant path length and the front-face (FF) mode where excitation is at an angle to the cuvette face and the light is reflected from the sample surface. These optical geometry differences also result in differences in the distance that light travels both when exciting the sample and when the emitted light “escapes’ the sample. These effects

have major effects on the spectral features and have unfortunately led to significant misinterpretations of data. The lack of acknowledging this has been quite common within applications to petroleum systems.

There are different strategies in minimizing some of these optical contributions by selecting different options in the radiation of the samples. However, many users of fluorescence spectroscopy seem to be unaware of this in most fields of sciences. [2,6] Front Face (FF) reflection spectroscopy is often used for more concentrated solutions with an expectation that FF has no self-absorption that decrease emission radiation. [7,8] If the optical density of the sample decreases, the penetration depth of the exciting radiation will increase and the impact of the self-absorption becomes increasingly important and will need correction before adequate information can be obtained from the measurements. [7,9] As the fluorescence radiation emitted exits the sample it will also be reabsorbed and the intensity of the recorded fluorescence will decrease. This apparent loss of excitation energy is called the inner filter effect. In the constant path length (RA), the long wavelength part of the spectrum is less affected by self-absorption compared to the front face mode in the case of crude oils and asphaltenes. But there are many other effects that can distort the recorded signal. In the investigation of concentration dependence, e.g. molecular association, the strategy should be to correct for these different effects as much as possible before interpretations. While fluorescence spectroscopy is a very sensitive technique in terms of lower detection limits it is also very much affected by many different effects which is increasingly important as one approach the concentrated regime. Spectral changes seen as red shifts with increasing concentration may be due to effects other than molecular association. E.g. inner filter effects in oil solutions are known to skew the spectrum by suppressing the short wavelength part of the spectrum more than the long wavelength part. For petroleum having basically a continuously decreasing absorption with increasing wavelength in UV-

vis this means that a significant redshift is observed which easily can and often has been wrongly interpreted as due to molecular associations.

Most fluorescence spectroscopy research as well as fluorescence based sensor systems utilize either constant emission wavelength or constant excitation wavelength techniques. For complex multi-fluorophore systems such as petroleum or coal liquids Synchronous Fluorescence spectra (SFS) may provide molecular resolution. In the SFS technique the wavelength region is scanned with a synchronous change in both emission and excitation wavelength with a constant wavelength difference ($\Delta\lambda$). Fluorescence emissions for a specific molecule are only observed if $\Delta\lambda$ matches the difference between an absorption band and an emission band. All other emission wavelengths are ignored (Figure 1). For multicomponent mixtures, SFS reduces spectral complexity and allows identification of specific compounds in the mixture.[10]

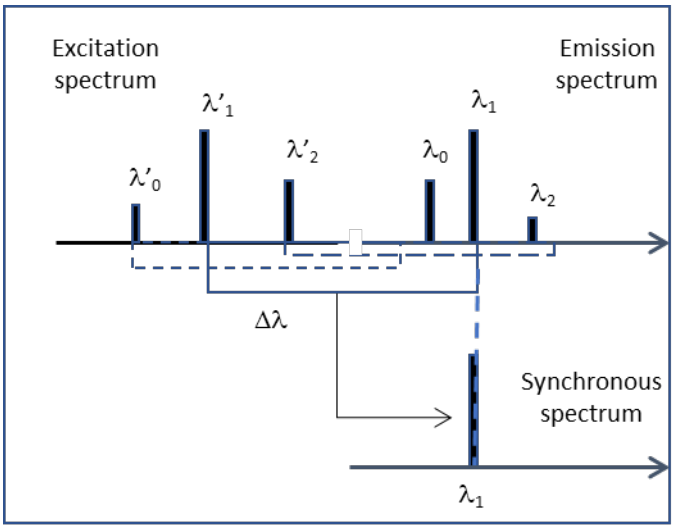

Figure 1. Mechanism of synchronous fluorescence spectroscopy.

Numerous quantitative and qualitative studies have applied fluorescence spectroscopy to petroleum chemistry and asphaltene association studies[8,11-15] These reports indicate red shifts to higher wavelength emissions at low concentration (< 100 ppm) and fluorescence signal/mass unit losses and deviations from linearity. These observations have been interpreted as due to molecular association or nanoaggregate formation. Recently, Zhang et al.[8] used excitation-emission (ex-ems) and time resolved fluorescence spectroscopy to investigate asphaltene aggregation in solutions from 0.1 to 10,000 ppm in toluene. They found by time-resolved emission studies that there seems to be different species aggregating at different concentrations and maybe the overall emission spectrum is dominated by non-associating species. Zhang et al.[8] also showed that the inner filter effect was important in the so called 90^{o} cuvette arrangement but concluded this was not present when using the front face procedure.

Vo-Dinh et al.[16,17] used synchronous fluorescence spectroscopy (SFS) to monitor and quantify very small concentrations of individual polyaromatics in solutions. John and Soutar[18] applied SFS to evaluation of various crude oils and the effects of concentration on spectral features relating these to molecular association. Merino-Garcia[19] used SFS reporting resin-asphaltene interaction observed as redshifts in fluorescence spectroscopy down to 2 ppm of asphaltenes in toluene. Da Silva Souza et al.[20] investigated effects of concentration with time-resolved fluorescence spectroscopy concluding that pre-aggregated or complexed asphaltenes were the excited species. They concluded the existence of significant association at 80 ppm in toluene inferred from the non-linear change in the spectral intensity with increasing concentration. Albequerque et al.[21] investigated asphaltene concentration effects between 16 ppm and 10 g/L , and as one of the few studies they applied inner filter corrections (see below). Goncalves et al.[22] investigated the association of asphaltenes using steady state fluorescence analysis but without applying any spectral correction. Daaou et al.,[23] examined sub-fractions of asphaltenes and the effect of

concentration up to 2 g/l (2000 ppm) discussing asphaltene association based on redshifts. Evdokimov et al., showed that changes in corrected fluorescence excitation-emission spectra may indicate that associations (e.g. dimerization) happens at concentrations as low as 0.5 ppm. [24]

A mechanism that affects quantification is the intensity quenching of fluorescence by energy transfer from one fluorophore to the next when the emission spectrum of one compound overlap with the exciation spectrum of another. This leads to emitted fluorescent light being used to excite lower energy molecules (larger fluorophores) increasing apparent emission at higher wavelength and diminishing emission at short. This is termed the Forster or Fluorescence Resonance Energy transfer (FRET). FRET is enhanced in multicomponent mixtures with multiple spectral overlap such as petroleum and asphaltenes causing the spectra to be virtually featureless broad bands.[25] Patra et al.[25] stated that Foerster Resonance Energy Transfer (FRET) is a major contribution to red shifts in fluorescence spectra of petroleum substances. This was mentioned as cascading of energy by Mullins and Wang in their early analysis of crude oil fluorescence. [26]

Before any observations of changes in fluorescence can be ascribed to molecular behavior one must have a clear understanding of the mechanisms affecting spectra and if possible correct for them either by using the right experimental methodology (geometry, concentration, etc.) or mathematically. There is a surprising lack of applications of these precautions in petroleum sciences as pointed out recently by Evdokimov et al [24], and these authors questioned the abundant conclusions in literature regarding association based on the unprocessed data. The present paper confirms this.

In the present paper we demonstrate how important spectral correction is before any conclusions can be drawn on the effects concentration in fluorescence spectroscopy and the mechanism behind any observed changes. We report fluorescence spectra of the Petrophase 2017 crude oil and it's n-heptane asphaltenes

as a function of two optical geometries (front face and right-angle geometry) and concentrations between approximately 0.2 ppm and 1000 ppm. We examine excitation spectra at 300 and 400 nm as well as synchronous scans. The aim herein is to investigate how and when correction may be needed, and how the corrections affect our interpretation of spectral data in the range between 2 and 1000 mg/kg toluene. Hopefully this can provide important guidelines to future research using fluorescence as a tool to understand molecular interactions in complex mixtures such as petroleum systems. The paper also points to the importance of doing all necessary corrections and data interpretations before any concentration dependent fluorescence behavior is concluded as being related to critical association behavior. The significance of the correction steps is illustrated by performing a series of classical quenching analysis with the aim to understand if molecular association such as nano-aggregation of asphaltenes can be the cause of the reported deviations from the behavior of non-associating/non-quenching systems.

EXPERIMENTAL

UV-vis spectra were recorded using a Cary 5000 spectrometer in 10 mm cuvettes solution of crude oil or asphaltenes in concentrations between 0.2 and 1000 ppm (wt.). These spectra are necessary in assessing and correcting inner filter effects in the fluorescence data. Results are displayed in Supporting material.

Fluorescence Spectra were recorded on a Perkin-Elmer spectrofluorometer LS55 with either the 90^{o} (right-angle) excitation to emission geometry or the Perkin-Elmer Front Face cuvette holder. Both Excitation-Emission and synchronous fluorescence spectra were recorded. Excitation wavelength was either 300 or 400 nm while recording emission from 200 to 900 nm at a speed of 200 nm/min.

Synchronous Fluorescence Spectra (SFS) were recorded with a wavelength difference ($\Delta\lambda$) of 20 nm in the same range and scan speed. 10 mm quartz cuvettes were used in both setups. All recorded spectra are shown in supplementary material. By using $\Delta\lambda$ = 20 nm significant effects of Rayleigh scattering are avoided.

The oil examined was the socalled Petrophase 2017 sample and the n-heptane asphaltenes (approximately 8 wt %) were provided in connection with the Petrophase 2017 project. A number of papers provide detailed descriptions of the precipitation as well as other properties of the Petrophase 2017 samples [27-31]. These samples were provided in a cross-laboratory study of various analytical techniques. The oil and the asphaltenes were used as received. Spectroscopic grade toluene was used as a diluent. Solutions were prepared by weight. Exact weights are reported in the following Table 1 but are in general referred to by approximate target concentration in ppm as also indicated. The use of the approximate concentration merely to make referencing in the text and figures easy to follow but also serves to describe ranges of concentrations.

All spectra not included herein can be found in the supplementary information.

Table 1. Concentrations of asphaltenes and crude oil in toluene

	True Concentration in toluene solution	
Concentration ID "ppm" Nominal – used in text.	Asphaltenes mg/kg	Crude Oil mg/kg
0.2 ppm	0.18	0.20
0.5 ppm	0.52	0.45
2 ppm	1.81	2.16

5 ppm	4.63	5.10
10 ppm	9.93	9.81
50 ppm	47.68	48.94
100 ppm	91.93	92.49
200 ppm	186.51	191.36
500 ppm	435.63	484.41
1000 ppm	983.16	1031.21

RESULTS AND DISCUSSION

Linearity in the fluorescence quantum yield is for non-quenched systems governed by a relation equivalent to Beer's law at low concentrations only valid at relatively low absorption (A< 0.1 Absorption units) :

$$Q = kI_0\left(1 - 10^{-\varepsilon l c}\right)\Phi \qquad [1]$$

The total emitted intensity Q is the intensity integrated over the entire spectrum which is related to the absorbed intensity I_a through $Q = kI_a\Phi$, where Φ is the fluorescence quantum efficiency and k is an instrumental constant. Q is related to concentration c through equation 1 where ε is the absorption coefficient, *l* is the path length, and I_o the incident light intensity. At high concentrations and high optical density Equation 1 states that the quantum yield or fluorescence intensity will be constant. In dilute solution Equation 1 can be approximated at single wavelength as a Beer's law relationship:

$$Q = k'\varepsilon l c I_0 \qquad [2]$$

This forms the basis for the initial analysis of fluorescence of petroleum solutions.

Three typical uncorrected spectra of the concentration series at right angle (RA), Front Face (FF) and Synchronous mode (SFS) is shown in Figure 2 with excitation at 300 nm of solutions of the asphaltenes as a function of concentration in toluene, as well as SFS spectra of asphaltenes. For the asphaltenes the red shift to higher wavelength and the suppression of the signal at short wavelength (below approx. 400 nm) is seen as the concentration increase. Due to the narrow range of fluorophores excited in in the SFS technique, as introduced above, the spectrum may carry more information about the molecular species involved. The use of SFS for quantification/identification of aromatics in petroleum systems has been discussed in detail in the literature. [16, 18, 32]

Spectra for the crude oil similar to those given in Figure 2 and asphaltenes are reported in supporting material S2-S5 . The concentration dependence is similar but less pronounced and shifted to higher concentrations.

These effects are much more pronounced in the RA geometry compared to FF and they are observed for both crude oil and asphaltenes. In both excitation and synchronous spectra, the short wavelength contribution disappears with concentration while a broad hump or peak appears around 500 nm. At long wavelength SFS intensities go through a maximum and decrease at high concentrations, while the short wavelength intensity decreases rapidly. These effects have previously been ascribed to formation of asphaltene aggregates and increased molecular interaction [22, 21, 8], but as we will show herein this is likely not the real reason for the observations.

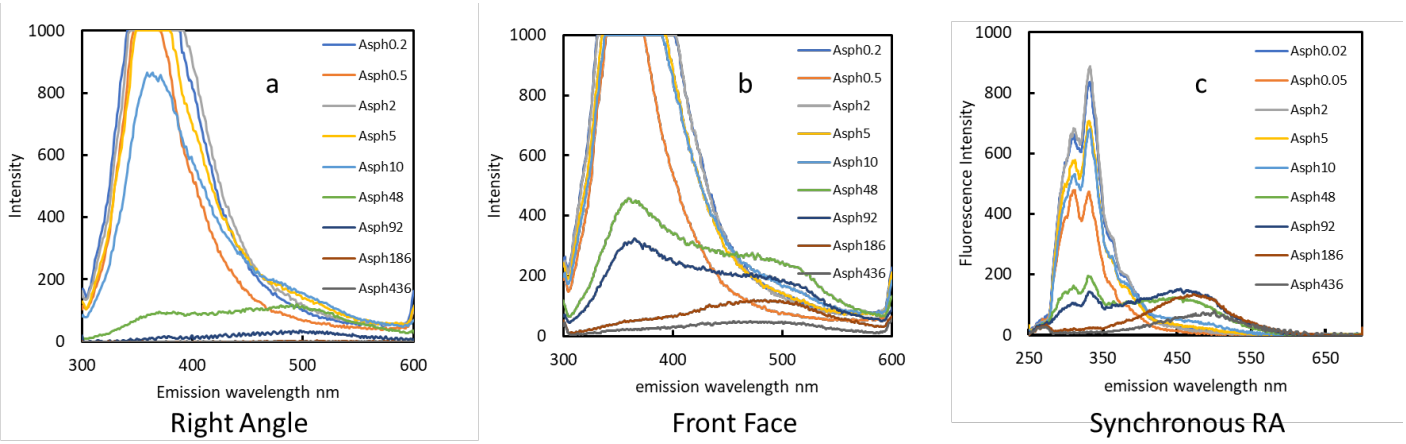

Figure 2. Spectra of asphaltene solutions in toluene showing the spectra obtained by a) right angel ex 300 nm; b) Front face ex 300 nm; and c) Synchronous mode at Right angle. Notice the large difference between RA and FF due to the difference in penetration depth.

The inner filter effect completely suppress the signal at short wavelength while a large hump appears at long wavelength as observed in Figure 2. As mentioned above synchronous spectra are known to carry more information about fluorophore sizes and specific spectral intervals have been correlated with the number of aromatic rings between 1 and 6+. Results of intensity variation at a specific short (335 nm) and long (500 nm) wavelength are shown in Figure 3 for SFS front face analysis of asphaltenes before correction of signals. The figure gives a typical example of how the apparent disappearance of signal from smaller fluorophores (335 nm) and appearance of peaks at longer wavelength (500 nm) can be interpreted as due to association into larger associated entities of lower energy and emission intensity.

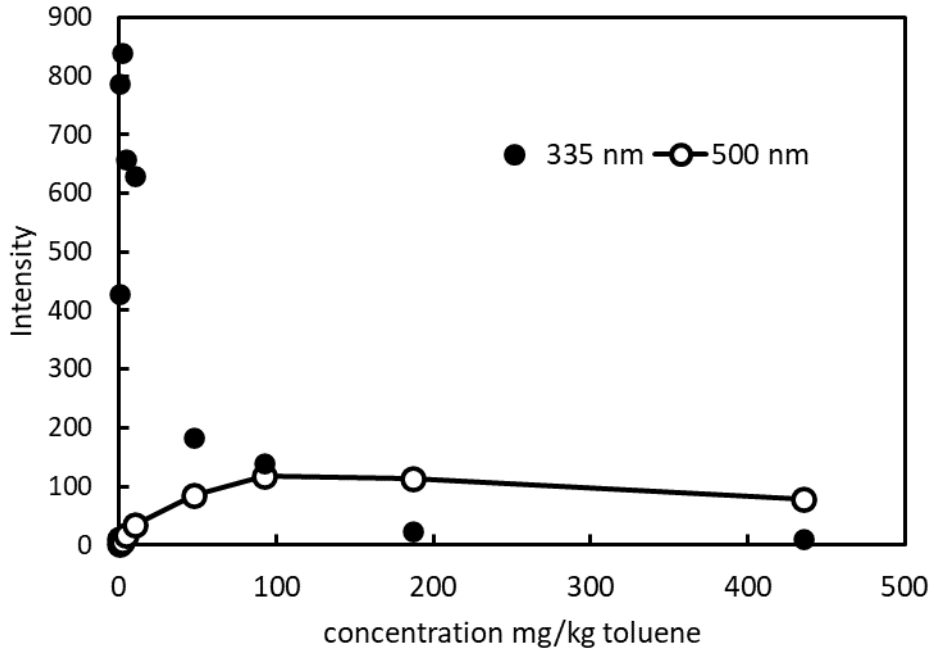

Figure 3. Asphaltene Front Face SFS Intensity at 335 and 500 nm as a function of concentration in toluene.

The above mentioned spectral changes of redshifts with increasing concentrations are observed in all our measurement performed of both asphaltenes and crude oil and can be found in the supplementary material. It should be noted that the oil solutions investigated are quite dilute in terms of the asphaltene content. Hence for crude oil the fluorescence redshift cannot be related to the asphaltene content alone. The above observed trend is common for asphaltenes and crude alike. For the crude oil, the apparent red shift is much reduced, and there is a nonlinear intensity-concentration relation leading to an intensity plateau at high concentrations which is evident from the similarity of spectra at 500 and 1000 ppm Figure 4. In Figure 4 we also observe the large difference in the redshift observed in the emission spectrum (400 nm) and the synchronous spectrum

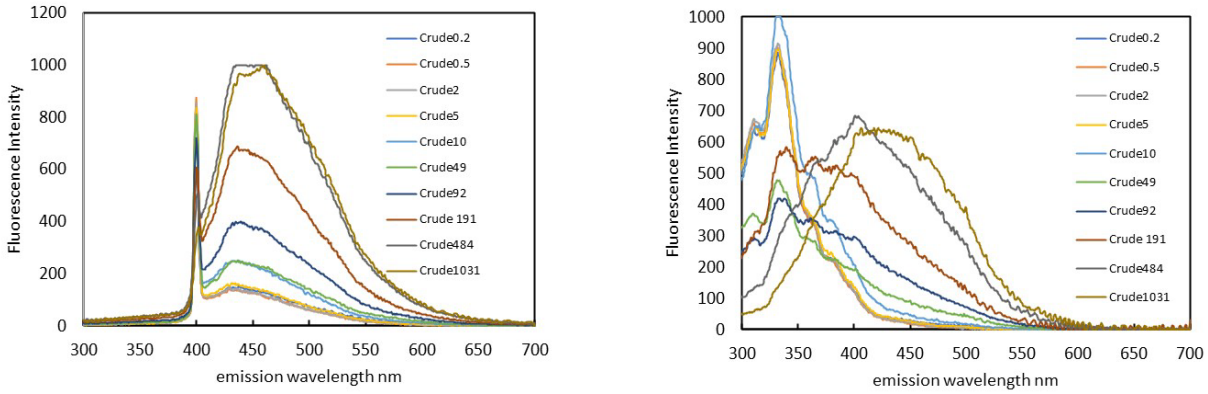

Figure 4. Excitation-Emission Fluorescence spectra (excitation at 400 nm, left) and synchronous spectra (right) of crude oil in toluene as a function of concentration in right angle configuration. SFS carries a significant amount of information compared to ordinary ExEms spectra. Notice the Rayleigh scattering at 400 nm in the ExEms spectra.

The frequently reported fluorescence intensity analysis of "association" is shown in **Figure 5** where the Front Face (FF) and Right Angle (RA) geometry are compared by plotting emission intensity at 440 nm as a function of concentration of asphaltenes of uncorrected data. In both cases we observe a maximum in the intensity which could erroneously been interpreted as a potential critical nano-aggregation related concentration effect as reported by many[17, 19 21, 22] . However, here the apparent shift (CNAC?) is observed at quite different concentrations of 10 and 90 mg/L for RA and FF respectively. For RA the decrease in intensity is much stronger and happens at a lower concentration. It is noteworthy that there is still an important spectral change in the FF sample. Hence one can conclude that the acclaimed lack of

inner filter effects in FF mode is not correct for these systems even at relatively low concentration. In the following we will show that spectral corrections will change the intensity-concentration relation significantly emphasizing the need of corrections in data analysis.

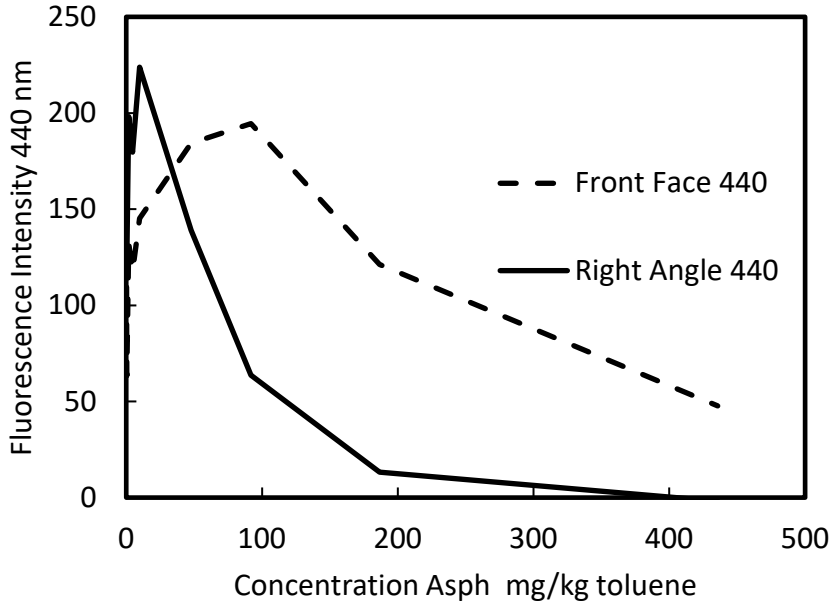

Figure 5. Comparison of fluorescence intensity changes at 440 nm for both FF and RA mode for excitation at 400 nm as a function of asphaltene concentration in toluene.

Inner filter spectral corrections

As indicated above there are obvious effects that need to be corrected for before any interpretations at a molecular level. As shown in the following, some of these corrections can easily be applied if the UV-

vis spectrum of the sample is available at the same concentration and per unit path length as in the fluorescence device. As anticipated and shown in supplementary material the UV-vis spectra of solutions of the crude oil and its asphaltenes show no significant features and an overall UV-vis Beer´s law compliance was found with linearity up to at least 3 A.U. for samples with concentrations beyond 2 mg asphaltenes/l toluene, Figures S1. Based on these measurements the fluorescence corrections were limited in concentration range for asphaltenes to below 200 mg/l and for crude oil the limit could up to 1000 mg/l. These limitations in concentration range depends on the excitation wavelength and the higher the wavelength the larger the concentration range span as the absorption coefficient falls off almost exponentially. See supplementary information for details.

Frequently the reported limiting absorbance value for proper fluorescence analysis is approximately 0.05 AU [33,7] for both FF and RA configurations. This cut-off is easily reached in solutions of asphaltenes and crude oil in common concentration ranges.[34,8] This rule of thumb limitation definitely excludes a significant portion of the work published in the literature. Other publications mention a cut off AU from 0.1 all the way up to about 1. This cut-off basically sets a limit to the concentration analysis somewhere below 50 ppm and for short wavelength even below 10 ppm or less. However, herein we apply it to all samples having a linear Beer's law relation in order to demonstrate and explore the correction effects across the entire range of concentrations examined in literature.

The apparent absorbance of the system may be a relation between molecular absorption and scattering of light by nano-aggregates. Hence the term should indeed be light extinction. Scattering and absorption can be very difficult to distinguish but we assume the effect of the two is basically the same as it is manifested by a decrease in the intensity of the recorded fluorescence signal. The main purpose of our present work is to investigate effects above 2 ppm. We observe linearity between concentration and

extinction (recorded as absorbance) for all wavelength below cut-off intensity. Therefore we allow ourselves to use the term extinction and absorbance as interchangeable, as the outcome will be the same in terms of simple inner filter corrections. We also take the linear behavior in the concentration range as an evidence that scattering and absorption contributions are proportional and that assume that there is no compensatin effect say of increased scatter being compensated by a non-linear increase in absorption.

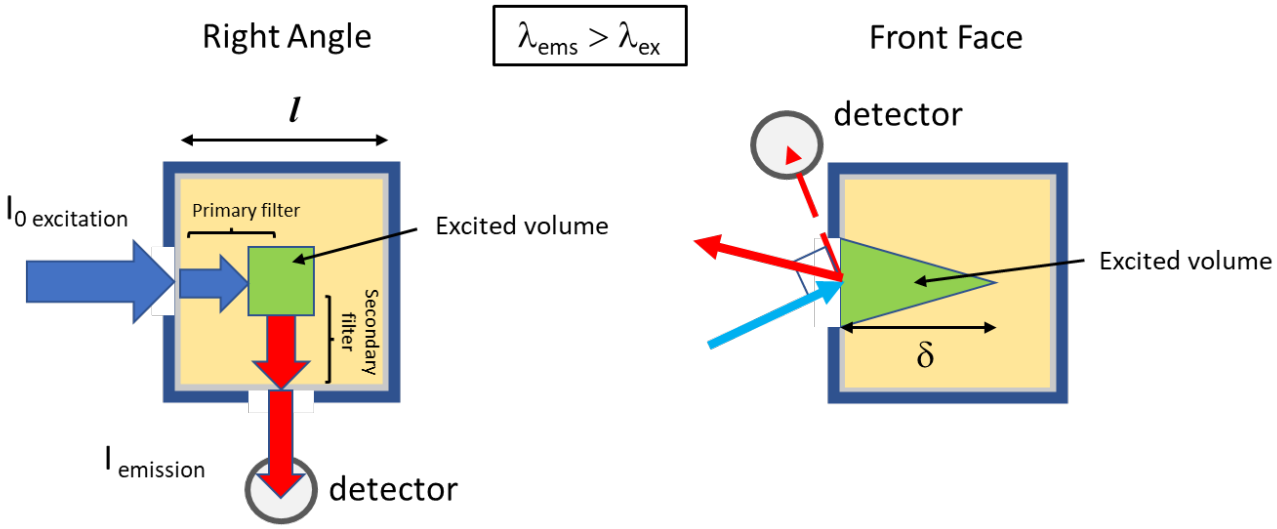

Figure 6. Elements of the inner filter fluorescence effect in right angle RA and front face FF configuration. The total path length is **l** for RA and 2δ for FF where δ is wavelength dependent.

The UV-vis spectral data is used for corrections for reabsorption of emitted light by the inner filter effect. Inner filter effects are corrected through mathematical treatments that are specific to the geometry of the sample container. It is assumed that fluorescence is only generated in a portion of the sample and both incident light and emitted light are affected by passing through sample volumes absorbing light intensity

as illustrated in **Figure 6** as primary and secondary filter for the typical right angle configuration. tThe Front Face optical geometry and sample penetration are also illustrated in Figure 6

The simplest correction for inner filter effects in a 10 mm 90º fluorescence cuvette configuration leads to an average pathlength of 10 mm: 5 mm of excitation (primary) and 5 mm of emission (secondary)

$$I_{corr} = I_{obs} * 10^{(Aex+Aems)/2} \quad [4]$$

A_{ex} and A_{ems} are UV absorbance in a 10 mm pathlength cell at the excitation and emission wavelengths, respectively. Spectral corrections are performed point by point.

In synchronous fluorescence spectroscopy (SFS) with small values of $\Delta\lambda$, A_{ex} is for petroleum systems close in magnitude to A_{ems} and either of these can be used with only minor differences when analyzing crude oil or asphaltenes with featureless absorbance spectra [1]:

$$I_{corr} = I_{obs} * 10^{Aex} \approx I_{obs} * 10^{Aems} \quad [5]$$

Figure 7 shows an examples of both the concentration effects on raw spectra and inner filter corrections of spectra of asphaltenes by both emission-excitation at 400 nm. As observed the correction is significant and the redshifts seen in raw spectra almost vanish. The noise is however obviously amplified.

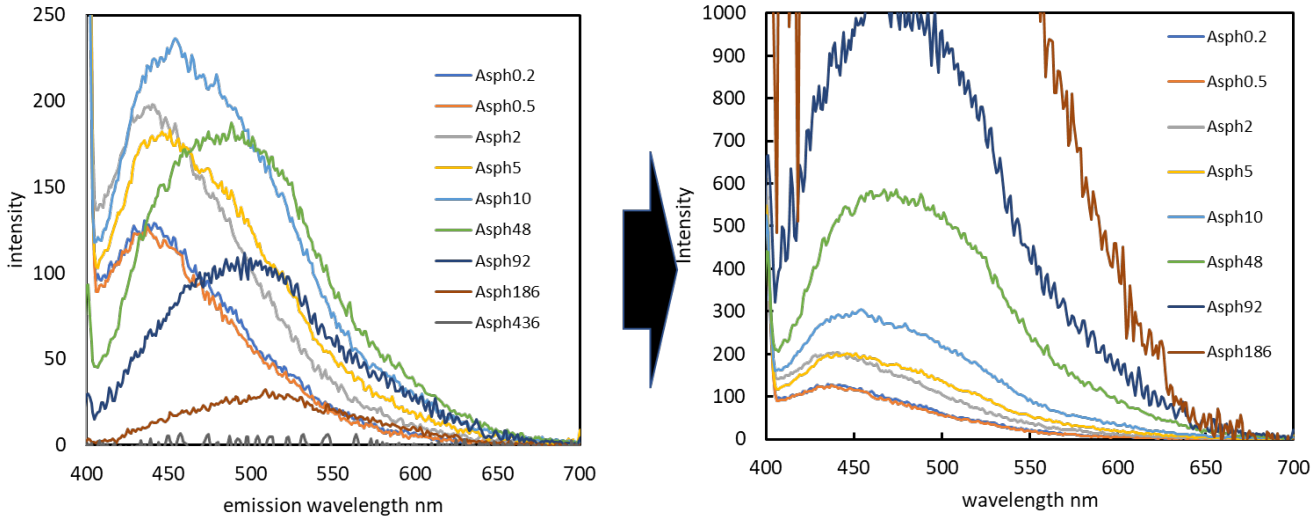

Figure 7. Inner filter correction of fluorescence excitation-emission (400 nm) spectra of asphaltenes in toluene at RA conditions. The 436 mg/kg toluene solution is not included in the left panel due to limited due to amplification of noise.

The asphaltene UV-vis absorbance spectrum is basically a featureless exponential decay function therefore corrections are most important at short wavelengths. Therefore the inner filter effect may dominant at the short wavelength but could be almost negligible at high wavelength. From this qualitative observation it is clear the lack of correction will lead to an erroneous conclusion on the dominance of larger fluorophores – for asphaltenes this could lead to erroneous conclusions on the presence of predominantly large polyaromatics and associated species.

Correction of Front Face spectra is more complicated due to the variation in penetration depth (δ) with wavelength. Figure 6. [34] In the present samples this effect is somewhat simplified by the constant decay in the UV-vis absorbance spectra which means that the penetration depth increase in a regular manner with increasing wavelength within the concentration range investigated. Ferreira et al. [33] reported a

relation between δ and UV-vis absorbance at unit length. If we assume the unit length to be A at a 10 mm pathlength this can be expressed as:

$$\delta = \frac{-\ln(1-f)}{2.303A} \qquad [6]$$

Where *f* is a correction function specific for the equipment geometry and settings and can be estimated by a sigmoid function of *A*. As an estimate of the penetration depth we can adopt the relation provided by Ferreira et al.[33] for the relation between *A* and *f* as a first approximation. While the cut-off absorbance for quantitative analysis is mostly given as 2.8 A.U., we have observed an upper Beer's law compliance of about 3.5-4 units. However, as Ferreira et al.[33] were searching for almost zero penetration to avoid issues with self-absorption they performed measurements at values going as high as almost 1000 A.U. At a value of 100 A.U. they still found a penetration depth of 0.09 cm. One way to confirm this is that solid samples attached to the back of the liquid cuvette can be easily measured through asphaltene solutions up to at least 100 ppm. As an illustration of this the Front Face penetration depths for the asphaltene solutions of 50 and 500 ppm in toluene as a function of wavelength were estimated using Ferreira´s approach. Figure 8. As seen there is a substantial penetration at the sample concentrations examined herein and it increases substantially with decreasing concentrations. Hence, we cannot expect the front face mode to be unaffected by inner filter effects for this type of samples. However, in the short wavelength region where the RA configuration is very much affected by inner filter, the effects for FF is substantially reduced as the correction is an exponent of δ(ex)*A(ex), as indicated in Equation 4 and 5. The increase in concentration therefore also leads to a relatively reduced inner filter in the FF mode

compared to the right angle mode. In the following analysis we assumed that the f-function given by Ferreira et al. can be adopted without any corrections for the difference in spectrometer configurations. While this may not be fully correct it is used here to illustrate the need of corrections also in the FF mode. The relation correlated to the data in Ferreira´s paper was $f = 0.4602 + 0.1655(\ln A) + 0.0165 (\ln A)^2$ which was correlated up to ln (A) = 0.85. See supporting material S6 for details.

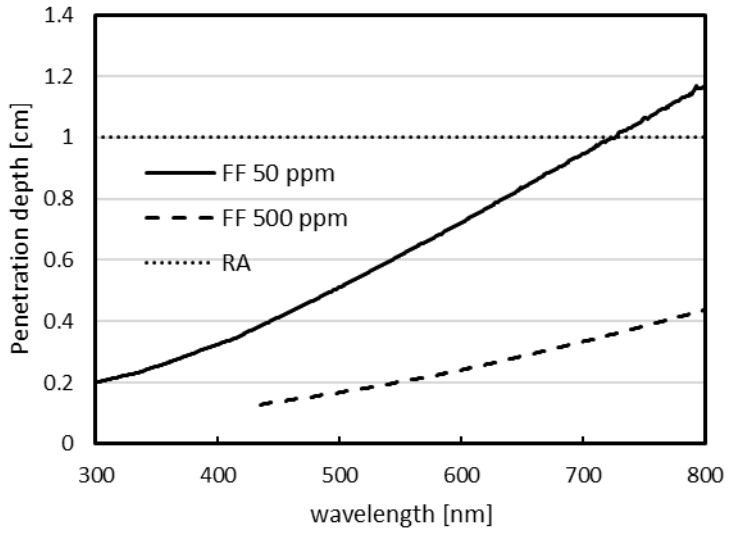

Figure 8. Estimated pathlength FF configurations as a function of wavelength. For FF given for two asphaltene concentrations of 50 and 500 mg/l. The RA pathlength is 10 mm or 1 cm.

Fluorescence spectral information and correction.

In the following we first demonstrate the effects of correction analysis on the simple RA geometry as the correction for inner filter effects are well documented and relatively easy, and more importantly with less assumptions compared to the FF correction methodology described. An example of spectral correction for SFS is given in Figure 9. As observed the correction leads to an almost complete recovery of the short wavelength emission spectra while a long wavelength hump is still seen from 50 ppm and up. Hence the illustration indicates the need of inner filter corrections prior to any conclusions derived from spectral changes while varying concentrations of either crude oil or asphaltenes as the correction is prominent.

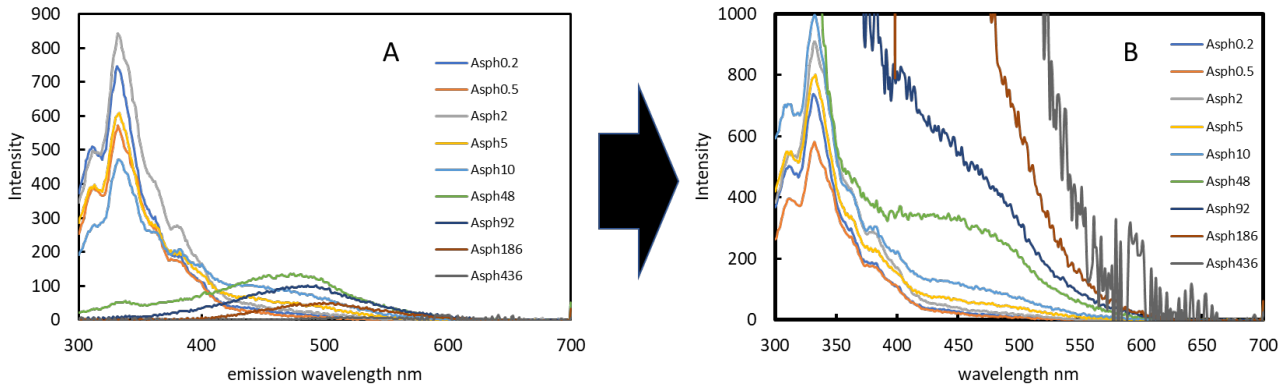

Figure 9. Synchronous RA spectra of asphaltene solutions before (A) and after (B) inner filter correction.

Based on the method of Ferreira et al, (29) the penetration depth was estimated and corrections using the correction factor f were tested for the simpler emission at 500 nm for excitation and both 300 and 400 nm and synchronous spectra. Figure 10. The adjustment of the total approximated path length (2δ) did not take the incident angle and the refractive index effects into account and therefore the path length and thereby the correction is somewhat underestimated. Despite the assumptions the analysis reveals that inner filter effects plays a role even in Front Face fluorescence analysis in the entire concentration range due to the finite and also sample dependent path length δ. Hence we conclude that FF mode not necessarily provide inner filter free spectra at elevated concentrations as suggested in many previous publications. Overall we observe that the inner filter effect completely suppress the signal at short wavelength while a large hump appears at long wavelength if corrections are not made.

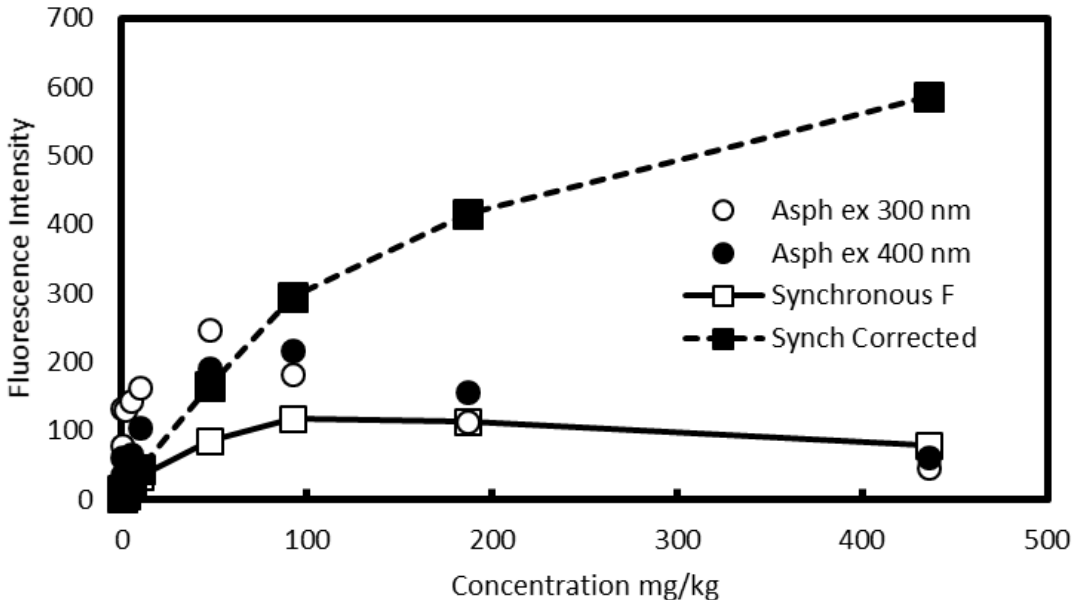

Figure 10. Asphaltene Fluorescence intensity at 500 nm in Front Face mode for excitation-emission spectra and synchronous fluorescence spectra (SFS). SFS correction using penetration depth according to Ferreira et al. [33]

As shown above the correction for inner filter effect is very much dependent on the penetration depth which is wavelength dependent and therefore this becomes rather cumbersome to correct for when numerous other effects are in play. This also includes the determination of the exact front face correction coefficient function f using calibrations standards as shown by Ferreira et al..[33]

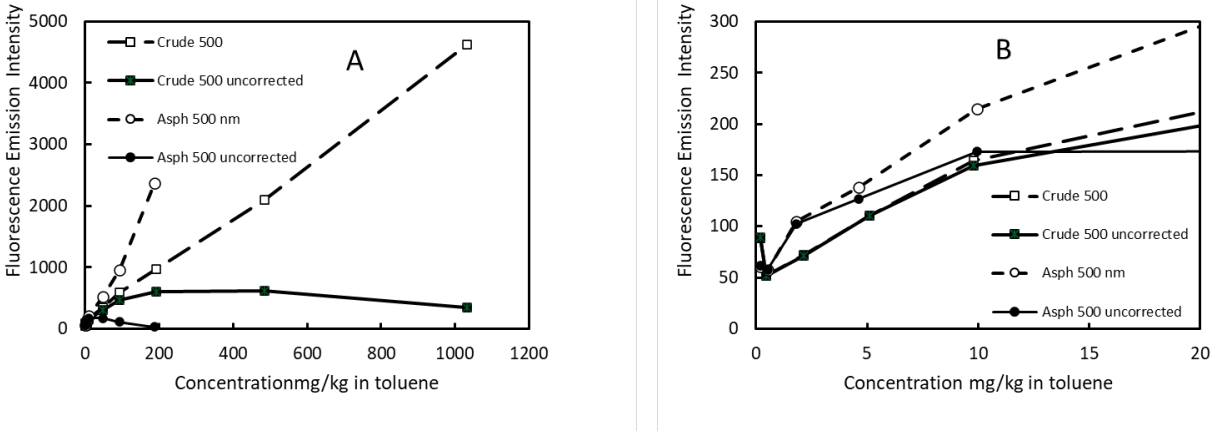

Figure 11. A: Effect of Inner filter correction on the emission at 500 nm in RA excitation (400 nm) spectra of both crude oil and asphaltene solutions. B: Zoom to enhance range below 20 ppm.

The effect of correction on fluorescence intensity at 500 nm for both asphaltenes and crude oil when excited at 400 nm is given in Figure 11. Emission at 500 nm was selected to increase the concentration range that can be analyzed based on the absorbance cut-off but the analysis gave similar results at other

emission wavelengths. Crude oil intensities were substantially affected by the correction, which reestablished an apparent linear concentration-intensity relation. Beyond 50 ppm the effect of correction is substantial for asphaltenes and the plateau often reported as evidence of aggregation disappears although the dependence is still not linear. For both samples a shallow break in the curve is observed at 10 ppm. Both samples show higher intensity for the lowest concentration 0.2 relative to 0.5 ppm as observed in the zoom panel of Figure 11. At such low concentrations the resulting non-linearity could easily be caused by tiny variations in both adsorption and fluorescence signal base line and noise levels as we are operating at very low intensities. The fact that we observe this for both crude oil and asphaltenes at the same concentration intuitively supports the cautious conclusion and clearly indicates that the observed break points and variations cannot be soundly concluded to be due to some critical association effect. We therefore refrain from extension of the analysis below 2 ppm even though data was available.

By comparison of normalized spectra (ex 400 nm, ems 440 nm) it is clearly observed that the spectra for crude oil falls in two concentration groups, one for concentrations at or below 10 mg/l and another with those above. In these two groups the normalized spectra are virtually identical. For the asphaltenes the spectra show a gradual redshift up to 100 ppm. While 50 and 100 ppm is almost identical the highest concentration of 200 ppm the spectrum blue shifts a little bit. At 10 ppm and above there is relatively more fluorescence intensity at longer wavelength in asphaltene solutions compared to crude oil and hence the results were coherent with the notion of higher content of large fluorophores in asphaltenes relative to crude. See Figure in supporting material

Overall there seems to be little information related to asphaltene association to be gained from direct observations of excitation-emission spectra even after correction. Effects at very low concentration

cannot unambiguously be used in a discussion of association as small errors are amplified by the correction procedure. As input to an analysis of critical concentration related phenomenon this may also be affected by background subtraction and small variations in concentrations during handling. We do not find that the accuracy of the present data can support such a discussion. Our caution regarding this is much in agreement with the findings of Zhang et al. [8]

The above data analysis after resolution of inner filter related effects leads to the question if the appearance of a peak at longer wavelength is more likely to be due to such effects compared to common suggestions that it can be explained by changes in the molecular environment of the fluorophores such as by association of molecules.

Nano-aggregate formation or not?

Throughout the present work the question arose whether or not the occurrence of a spectral contribution at longer wavelength represents formation of nano-aggregates or not. Association has been inferred in many fluorescence works on asphaltenes but without the appropriate correction for inner filter effects. As observed simple inner filter corrections may remove the concentration effect making it obvious that previous conclusions regarding critical nano aggregate concentrations (CNAC) based on large shifts in uncorrected spectra should be disputed. Also a redshift after correction may still not be an indication of true aggregations and can be the result of just proximity of molecules as the concentration increase providing another energy pathway. The principle of resonance energy transfer from overlapping absorbance and emission spectra, FRET, is one competing mechanism which will lead to enhancement of emission at longer wavelength on the expense of the emission at short wavelength. Energy transfer is

particularly a problem with multicomponent mixtures as they provide a multitude of pathways even by the transfer of energy through several molecules with overlapping spectra.[1,26] The doubt whether association is indeed a main factor in the spectral changes is corroborated by the observation of red shifts in solutions of crude oil having similar concentration dependencies as that of the asphaltenes especially as the crude oil has an asphaltene concentration of only about 8 wt. % . That would mean the critical concentration effect should be moved approximately 12 times higher in the crude oil relative to the asphaltenes assuming that the alleged CNAC is not just a phenomenon observed in solutions of separated asphaltenes but could also happen in toluene solution of crude oil independently of other crude oil components.

A critical concentration for aggregation of asphaltenes (CNAC) above a certain concentration would suggest that all molecules added in excess of CNAC are in the aggregated state and the due to energy transfer amongst associated molecules this would lead to significant fluorescence quenching.These aggregates are expected to have emission at a longer wavelength if not completely quenched by the aggregate formation as known for pyrene solutions. That would under ideal conditions result in a constant contribution at low wavelength as there should be a constant concentration of non-associated species in equilibrium with aggregates observed at long wavelength. Non-radiative effects would increase and overall intensity would therefore drop for the aggregated species. It has been argued that if these aggregates are formed by face-to-face stacks of large aromatic sheets the quenching would be very efficient.[8] Zhu and Mullins[35] mentioned non-fluorescent dimers or complete fluorescence quenching of dyes such as Rhodamine B and porphyrins. If the mechanism is tail-to-tail association the quenching is only partial. Both mechanisms and combinations can easily be present in such a varied material as the

asphaltenes. The corrected spectra still show some degree of reduced intensity with increasing concentration.

The energy transfer by the FRET may best explain the observations of departure from linearity after inner filter correction as FRET serves to boost signals a long wavelength on the expense of radiation at short. FRET has a clear relation with concentration if donor and acceptor are independent molecules. There are also examples of intramolecular FRET when donor and acceptor are part of the same molecules, but independent in terms of fluorescence[36] . This could for asphaltenes be a naphthalene ring system covalently bonded but not conjugated with a larger ring system as sometimes depicted in the literature (14). The distance requirement between independent molecules for FRET to efficiently affect spectra is below approximately 10 nm. If assuming same size molecules and a nominal asphaltene molecular weight of 1000 g/mole this is not reached before the concentration is somewhere between 100 and 1000 ppm depending on the approach taken in the estimation of the distance. If the molecular weight is lower or if there is a substantial amount of smaller polar molecules present, the molecular distance at a given concentration will decrease and favor FRET. Localized association dynamics will obviously lead to increased proximity and FRET will along with other quenching phenomena occur in these cases.

The FRET analysis will normally require well resolved peaks for both donor and acceptor [1] and a relative FRET efficiency calculated from ratios of intensities of the two. This is not possible in petroleum fractions without addition of an external donor. Hence another option that we suggest in analysis of the extent of FRET is to look at the independence of the emission intensity in a long wavelength contribution when changing the excitation wavelength as suggested by Yokota et al.[11]. If the sample is excited at 300 nm this will lead to fluorescence from small molecules. This energy ca be transferred efficiently to larger molecules given the concentration is high enough. When the sample is excited at 400 nm, the energy

from small molecules is not released and therefore energy is not transferred. Therefore we expect a decrease of the emitted light at longer wavelength relative to the 300 nm excitation case. In other words, if the ratio of intensities emitted by excitation at the two wavelength is constant FRET is not an issue. If FRET occurs then the emission by excitation at 300 nm should be larger than the emission by excitation at 400 nm and the ratio between these should increase with increasing FRET contribution. If the concentration of aggregates increases with total concentration relative to the "monomer" concentration as implied by a critical phenomenon this would mean that excitation at 300 nm should have a lesser effect as the concentration of the free molecules should diminish relative to the aggregates that are excited at 400 nm. Hence the emission intensity after excitation at 300 nm should be less relative to the intensity at the excitation at 400 nm. What we observe is overall an increase in the intensity ratio, Figure 12, and therefore FRET indeed seems to provide an important contribution to the distortion of asphaltene and crude oil spectra. FRET is even more effective in these systems by cascading energy through series of donors and acceptors where some molecules serve as both donor and acceptor [25,26]. The FRET mechanisms can be very active in both asphaltenes and in the crude oil as has been shown by Patra et al. [25]. The relatively higher concentration of smaller fluorophores in the crude oil will here work as an amplifier of the emission from the bigger fluorophores resulting in the red shift. As FRET effects are almost impossible to remove this sets a certain boundary on any interpretation of data in terms of quenching either by collision of molecules or by actual molecular interaction also known as static quenching. However, to complete the present data analysis we apply common approaches to quenching analysis and discuss the issues involved in analysis of petroleum multicomponent systems.

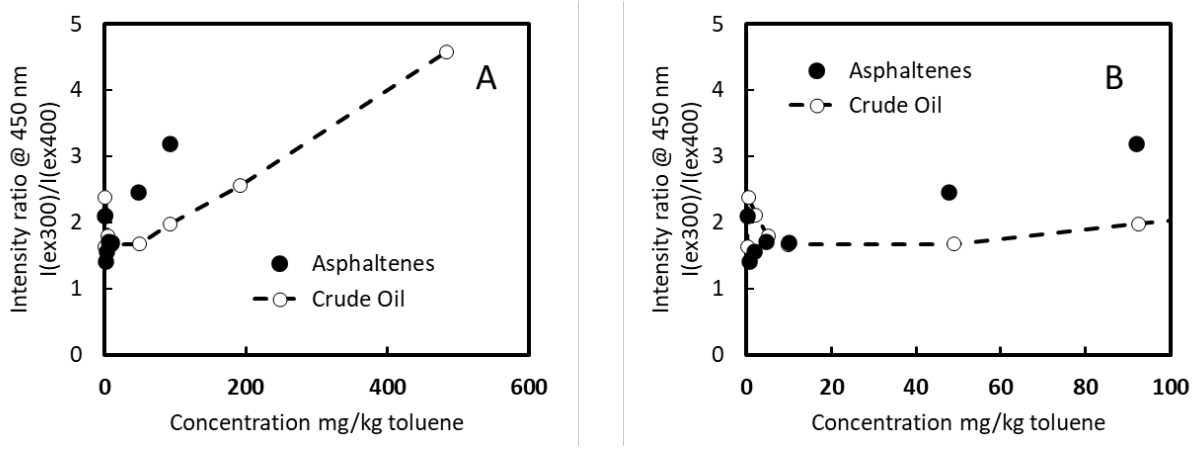

Figure 12. Effect of concentration on the Energy Transfer (FRET) analyzed by excitation at 300 and 400 nm and emission at 450 nm for asphaltenes and crude oil solutions in toluene (A). (B) is a zoom of the region below 100 ppm showing the non-regular behavior below 10 ppm.

Can classical Quenching analysis revealing of association?

Real quenching is the enhancement of the non-radiative decay routes from the excited state to the ground states. This may be observed for many systems including polyaromatic systems. For associating systems, we will only consider two quenching mechanisms: formation of ground state dimers and excited state dimers. Formation of ground state dimers will lead to a decrease in monomer concentration and as dimers absorb light at higher wavelength a red shift will be observed. While quenching is a problem for fluorescence spectroscopy as a quantitative tool, quenching analysis is extensively used in biotechnology and to some extent in petroleum sciences to understand intermolecular interactions.[1, 21, 26] Quenching is normally reported by plotting fluorescence intensity of a given solute at fixed emission wavelength or total fluorescence across a wavelength range versus solute concentration. Quenching is then manifested as the deviation from a linear concentration dependence and this has for asphaltenes often been assumed

to be due to association of molecules. However, the relative intensity decrease observed with increasing concentration may have different causes. Quenching could stem from collision and subsequent energy transfer to other molecules; from true association; from the increased absorbance of both the incident energy that excites the fluorophores as well as the re-absorbance of the emitted fluorescence light. Most frequently it is a combination of all the these. One crucial first step in the analysis is the correction for inner filter effects which should not be neglected in any fluorescence data interpretation.

To further explore the potential of fluorescence in pinpointing association of molecules a brief investigation is conducted on classical quenching analysis approaches. Real quenching occurs due to close encounters between a quencher molecule and a fluorophore through collision, association or specific interactions. If a quencher is present the relation between quencher concentration C and relative fluorescence intensity at a given wavelength or the total emitted fluorescence (integrated signal) follows the simple Stern-Volmer Equation[1]:

$$\frac{F_0}{F} = 1 + K[C] \qquad [8]$$

F_o is the radiation in the absence of quencher and F is the radiation at a given quencher concentration [C]. The constant K is a combination of all rate constants of quenching mechanisms (collision, static etc.). The magnitude of K indicates the efficiency of quenching or the binding strength of quencher and fluorophore. The Stern-Volmer equation is normally used to analyze dynamic quenching by changing the quencher concentration C. Equations 8. A linear relation after all spectral corrections is consider to represent collision quenching as the main mechanism. Several systems are reported with deviations from linearity and SV analysis may show both upwards and downwards curvature [1, 4]: If both static and dynamic quenching appears the curvature of the SV-plot of F_0/F vs C is generally positive and upwards.

On the other hand, if the quencher cannot get access to the fluorophore due to molecular re-arrangements at high concentrations quenching is less pronounced leading to downwards curvature. [1, 4]For complex systems it can be very difficult to separate the contributions especially with the steady state fluorescence technique applied herein.

Self-quenching has been reported for many simple systems where changes are observed in spectra as a function of solute concentration with no added quencher.[3, 10, 36, 37] Self-quenching may be attributed to the above mechanisms, the interaction of ground state and excited state molecules, or to internal FRET leading to a decrease in intensity with concentration after all appropriate corrections.[36]

In petroleum analysis "self-quenching" appears, either through encounters of like molecules (e.g. asphaltene-asphaltene) or with "other molecules" (e.g. in crude oil: resin-asphaltene). There is almost a continuous overlap of individual emission spectra of one type and excitation spectra of other types leading to perfect conditions of energy transfer not necessarily reflecting aggregation or associative behavior. In the present case the increase in total concentration leads to a proportional increase in both "quencher" and fluorophore concentration. The assessment of the un-quenched fluorescence yield F_0 therefore requires some assumptions for such systems. While the quencher concentration is proportional to the total concentration the actual quencher concentration is unknown. Therefore the quenching constant K may not be meaningful except for comparison. In order to partially overcome this obstacle the analysis may be based on the total fluorescence quantum yield scaled to the concentration: $F = Q/c$ and $F_o = Q_o/c_o$.[21]

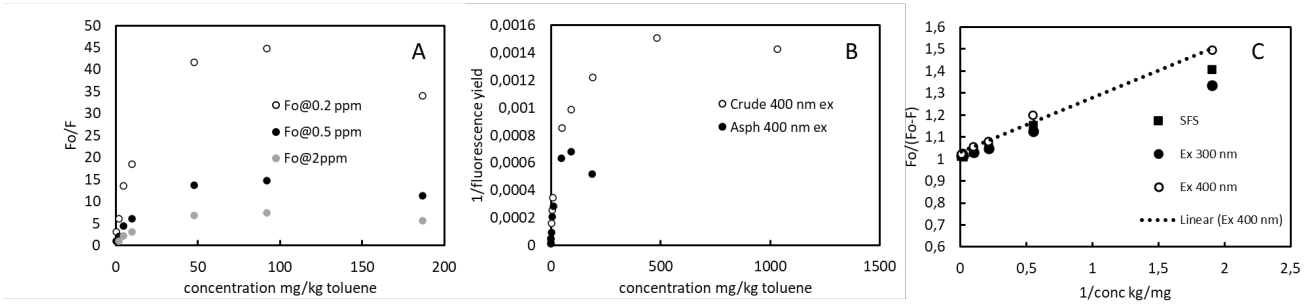

Figure 13. A: Stern-Volmer plot (Equation 8) for asphaltene excitation spectra (400 nm) showing the effect of selection of spectral definition of F_0. B: SV analysis acc. Equation 9 for both crude oil and asphaltenes. Excitation at 400 nm and integrated yield in the interval [410;700]. C: Analysis of fractional quenching of asphaltene fluorescence intensity acc. to Equation 10 showing no shielding of fluorophores as the intercept $1/f_a = 1$.

We report this analysis for 400 nm excitation spectra as similar results were obtained for all geometries and 30 nm excitation. Analysis of specific wavelength intensity or total fluorescence yield also provided similar relations between concentration and fluorescence intensity/concentration. Regardless of the different relative responses obtained the overall result for all cases is a downwards negative deviation as seen in Figure 13A. The lowest concentrations analyzed were assumed to be virtually quenching free and gave similar results when used as F_0. This negative curvature was also reported by Albuquerque et al. [21] for two asphaltenes in toluene solution between 16 ppm and 10 g/L.

Albuquerque et al. [21] also suggested an alternative formulation to avoid the uncertainty of F_0 estimations for asphaltene self-quenching analysis. Equation 9. This approach will not give any qualitative difference in the analysis of data and herein also showed negative curvature for all samples.

$$\frac{1}{F} = \frac{1}{F_0} + \frac{K}{F_o}[C] \qquad [9]$$

The negative curvature was by Albuquerque et al. explained as quenchers being removed from the solution by association of molecules [21]. However, that would be indicative of a static quenching which would result in an upwards curvature. Our results resemblance protein-quencher interactions where fluorophores get masked and therefore inaccessible to the quencher due to conformational changes as the concentration increase [1,4] . This could be a plausible explanation especially in the view of nano-aggregate formation. Aqueous micellar solutions have also been reported to show a downwards trend as the overall fluorescence becomes a combination of fluorophores in the solvent and in the interior of the micelle as well as the partitioning and binding with the quencher in the two phases.[22] One could despite the relatively small size of the asphaltene nano-aggregate assume that the aggregate in part could act as pseudo-phase with inclusions of masked quenchers.

However, SV plots of both crude oil and asphaltenes show negative deviation from linearity using equation 9. Figure 13B. The break point where 1/F for excitation at 400 nm goes through a maximum is translated to approximately 5 times higher concentrations for the crude oil solutions relative to asphaltene solutions. Given the asphaltene concentration is in about 8 wt %, this would imply that fluorescence quenching happens more readily in crude oil solutions compared to asphaltene solutions. Reaching a plateau means that there is no effect of concentration or quencher. While we could claim this to be due to association this would not be an unbiased answer as the basic conditions for the SV approach is not fulfilled. We therefore cannot claim typical quenching phenomena to be responsible for the decrease in F_o/F or 1/F at relatively high concentrations as the obervation corresponds to a relative increase in fluorescence intensity. Even when applying spectra correction the observation could stem from an over-

correction especially at the short wavelength where A is large. When performing the SV analysis by excitation at 400 nm the plateau is reached but no decline is observed. Hence this outcome indicates that excitation at 300 nm leads to a relative increase in fluorescence at high concentration where there is a high probability of both collision, static association interactions and just mere proximity of fluorophores. The latter seems to be the only viable explanation and hence this means FRET could be an overriding mechanism in the spectra of these samples at elevated concentrations. We would expect association of asphaltenes to also lead to a true quenching of fluorescence from the individual asphaltenes. This analysis is independent on whether we use total fluorescence or specific wavelength emission.

To finally investigate another option which is the shielding effect by molecules being inaccessible we turn to a theory devised for protein analysis.[1] When proteins fold part of the fluorescent entities gets inaccessible. The fraction of accessible sites (f_a) for quencher analysis is frequently analyzed based on

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a K_a}\frac{1}{C} + \frac{1}{f_a} \qquad [10]$$

Here K_a is the effective SV quenching constant. By plotting $F_o/(F_o\text{-}F)$ vs 1/C we can get an indication of the fraction of accessible interaction sites on the asphaltene molecules relative to the total amount of interaction sites. The intercepts of this analysis for both oil and asphaltenes are very close to 1. Hence for both crude oil and asphaltenes this analysis fails to provide any indication of an important effect of the shielding of quenching sites by conformation changes such as association. Figure 13C. The result of this analysis again could be due to the condition of the experiment where the ratio of quencher to fluorophores is constant. All data show the same trend and we did not see any substantial variation in f_a as determined from the intercept.

As a conclusion the quenching analyses applied herein on petroleum systems appear to fail as the complexity and especially the multiple overlap of absorbance and fluorescence bands dominate these otherwise simple approaches. Therefore, it seems that without addition of a well-known quencher one may not be able to fully understand the observed trends by the SV analysis.

In most analysis of asphaltenes it is assumed that there is no effect of any distribution between fluorescent and non-fluorescent asphaltenes although we know that asphaltenes may contain even larger proportions of the latter. However, we do not know if these are highly quenched associated asphaltenes or simply molecules with very low quantum yields due to lack of larger conjugated aromatic ring systems [8, 37] . It remains a challenge how we can incorporate this into our understanding of the fluorescence signals and how the distribution of these may affect the association of asphaltenes.

As indicated in the above analysis the vastly unknown and very complex composition of the fluorophores of asphaltenes and crude oils highly hampers the full use of the fluorescence spectroscopic technique. One possible way of better elucidating the mechanisms involved in the signal quenching would be to add well known compounds and concentrate on the fluorescence of these. In an unpublished study we used 1-methyl-naphthalene to understand the energy transfer and quenching mechanisms in diesel blends leading to better understanding colors of blended products. One may also add larger aromatics such as phenanthrene which potentially could engage in actual molecular association with asphaltene by possible pi-pi interactions. However, adding tracers was outside the scope of the present work and we are not aware of any such published research aiming at understanding quenching in petroleum systems.

CONCLUSION

We have thoroughly analyzed the concentration effects in fluorescence spectra of both "petrophase 2017"asphaltenes and crude oil using the two classical optical geometrical options, Front Face (FF) and Right Angle (RA) excitation-emission. The former is shown to compensate for inner filter effects only at short wavelength, where there are substantial effects in RA configuration across the entire spectral range. Inner filter corrections have unfortunately not been the practice in many studies of petroleum fractions and conclusions drawn from those studies on association of asphaltenes are not trustworthy. We find that inner filter corrections are not important below approximately 10 ppm for both crude and asphaltenes in either FF or RA. At elevated concentrations and after inner filter corrections the substantial "quenching" at short wavelength is basically removed although some intensity decrease is still seen with increase in concentrations. Corrections for the very high concentrations (500-1000 mg/kg) leads to large noise levels due to the very low intensity of the raw signal at both long and short wavelength. To understand if observations are due to static (association) or collision quenching a Stern-Volmer (SV) analysis was made. The SV analysis shows that classical association cannot be the explanation, and conformational changes and/or blocking quenching sites seem not to be the prevailing reason despite similar reports observed for protein fluorescence. Therefore, the most important effect leading to distortion of the spectra resembling red shifts known from molecular association seems to be intermolecular energy transfers such as FRET which in particular takes place at high concentration. For FRET to occur physical proximity is important (<10nm) between molecules. At the high concentrations, we observe clear indications of FRET as responsible for the increase of fluorescence at high wavelength on the expense of fluorescence at short wavelength.

One important point to conclude from the work reported herein is that the multi absorber-fluorescer or donor-acceptor nature of petroleum means that a simple analysis such as Stern-Volmer in reality is not

applicable in the analysis of concentration dependence. In FRET this means that energy may likely be transferred through several molecules with decreasing energies therefore eradicating all emission at short wavelength and substantially boosting emission at long wavelength. However, the more links in this chain of molecules the less energy will reach the larger molecules. This nature of the sample also affects the application of classical quenching analysis with substantial assumptions when applied to petroleum.

Association such as nano-aggregation is not out-ruled by these observations and there is indeed a change in intensity after inner filter correction at low concentrations where spectra should be unaffected by FRET. We also observed for both excitation spectra and synchronous spectra and in both FF and RA mode that low concentration intensities (below 5 ppm) is not showing a linear relation with concentration. However, we did not set systematic changes in spectra which could show the presence of "monomeric" asphaltenes as reported by Endomikov et al.[24] And the number of measurements in this concentration range seem too low to allow for a proper explanation of the non-linearity in the low concentration region where both fluorescence signals and absorbance spectra have very low intensity.

Stacking of large aromatic sheets would lead to energy transfer between molecules causing an increased significant quenching contribution and loss of fluorescence from the aggregates as energy is dissipated across the molecules in the aggregates and released as non-radiative energy. Therefore association could actually be expected to lead to a decrease also in the long wavelength contribution this is observed not to happen in any substantial way after inner filter correction of any series observed herein

One important point to conclude from the work reported herein is that the multi absorber-fluorescer or donor-acceptor nature of petroleum means that a simple analysis such as Stern-Volmer in reality is not

applicable in the analysis of concentration dependence. For FRET this means that energy may likely be transferred through several molecules with decreasing energies therefore eradicating all emission at short wavelength and substantially boosting emission at long wavelength. However, the more links in this chain of molecules the less energy will reach the larger molecules. This nature of the sample also affects the application of classical quenching analysis with substantial assumptions when applied to petroleum.

Overall, the above shows that fluorescence spectroscopic analysis of even diluted petroleum fractions may be highly affected by numerous effects that we at present cannot separated. Therefore concentration studies may only provide a glimpse of the molecular interactions in the solution leading to e.g. nano-aggregate formation. We encourage studies of true donor-acceptor interactions where the donor is a well-known single compound.

ASSOCIATED CONTENT

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting information. Additional data and supporting material is available as: UV-vis (S1) and all recorded Fluorescence spectra including innerfilter corrections S2-S5, as well as the Ferreira f-factor calculation (S6) and a brief discussion of ratios of aromatic type determination from synchronous fluorescence spectroscopy (S7).. The supporting information is available free of charge via **internet at XXXXXXXX.**

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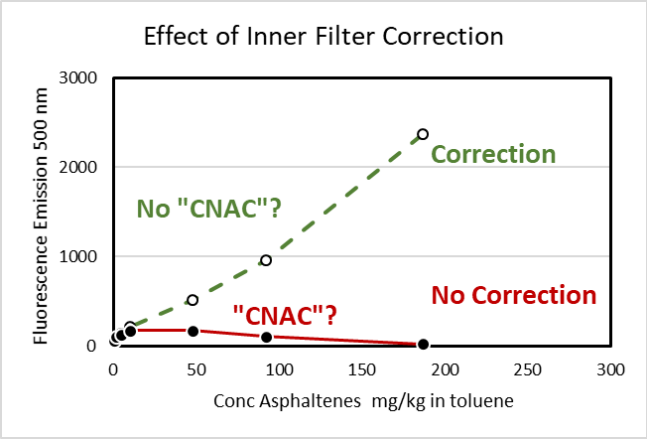

ABSTRACT FIGURE.