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Application of a high-performance liquid chromatography fluorescence detector as a nephelometric turbidity detector following Field-Flow Fractionation to analyse size distributions of environmental colloids

F. v.d. Kammer [a,*], M. Baborowski [b], K. Friese [b]

[a] *Department of Geological Science, Environmental Geosciences Group, Vienna University, Althanstrasse 14, A-1090 Wien, Austria*
[b] *UFZ-Centre for Environmental Research, Department for River Ecology, Brückstr. 3a, D-39114 Magdeburg, Germany*

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Abstract

A new operation mode for HPLC-type fluorescence detectors is presented and evaluated using synthetic and environmental particles in the colloidal size range. By applying identical wavelengths for excitation and emission a nephelometric turbidity or single angle light scattering detector is created which can be easily coupled to flow or sedimentation Field-Flow Fractionation (Flow FFF or Sed FFF) for the analysis of colloidal dispersions. The results are compared with standard UV–vis detection methods. Signals obtained are given as a function of particle size and selected detection wavelength. Conclusions can be drawn which affect the current practice of FFF but also for other techniques as groundwater sampling and laboratory column experiments when turbidity is measured in nephelometric mode and in small sample volumes or at low flow rates.

Keywords: Field-Flow Fractionation; Turbidity; Nephelometry; Environmental particles; Concentration detector; Fluorescence detector in nephelometric mode

1. Introduction

1.1. Environmental colloids

Naturally occurring particles in the nanometer size-range (natural colloids) are playing a significant role in environmental processes [1–4]. Due to their relatively large specific surface area, contaminants with low solubility in ground and seepage waters can be adsorbed predominantly to surfaces of natural colloids. If these colloids are or become mobile in the subsurface contaminant transport may be enhanced significantly [5–7]. The increasing efforts to understand contaminant behaviour (transport, partition, bioavailability) in the presence of natural colloids and to collect field data for evaluation of laboratory experiments are hampered by the lack of suitable methods for the analysis and characterisation of natural colloids [8].

From about 1987 Field-Flow Fractionation (FFF) was introduced for the analysis of natural colloids when the first instruments became commercially available [9,10]. FFF proved to be very powerful in separation, sizing and characterisation of natural colloids, especially together with a coupling to multi-element detection systems as, e.g. ICP-MS or ICP-OES [11–14].

1.2. Field-Flow Fractionation (FFF)

Flow and Sed FFF are chromatography-like separation methods relying on the interaction of hydrodynamic and centrifugal forces with macromolecules, colloids and particles and without the utilization of a stationary phase. In first place and according to underlying theory, the method enables the continuous separation of molecules and particles in relation to their size-related properties (Fig. 1) [15]. The retention of colloids in the FFF channel is a function of their diffusion coefficient/Stokes diameter (Flow FFF) or buoyant mass (Sed FFF).

The theory and application of FFF is described in detail in [15] and with emphasis on the analysis of natural colloids in [11].

However, when FFF is used for the size distribution analysis of natural colloids the respective equivalent particle size is derived from retention times of the particles in the channel and

* Corresponding author. Tel.: +43 1427753380.
E-mail address: frank.kammer@univie.ac.at (F. v.d. Kammer).

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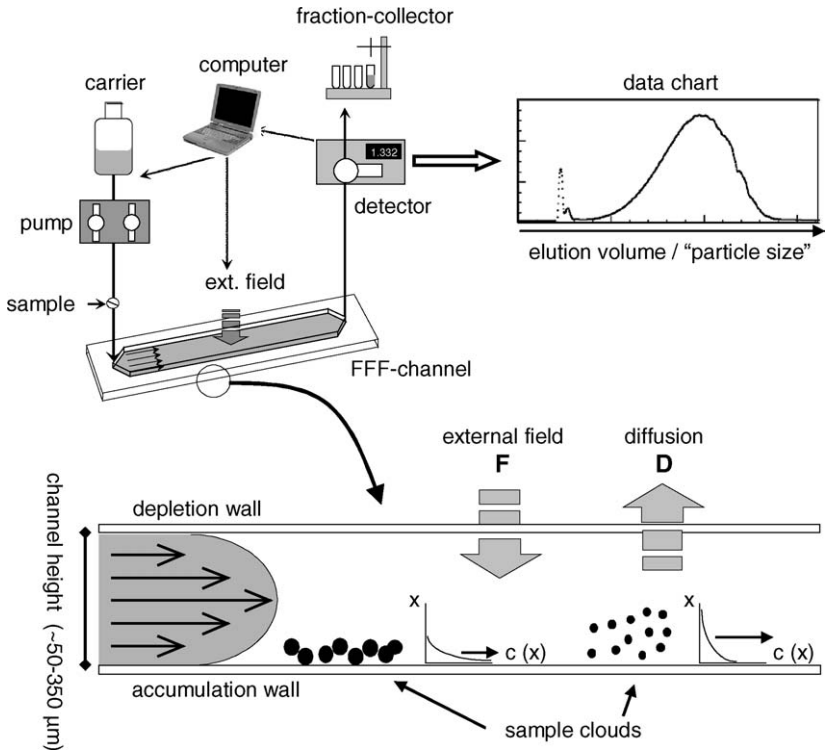

Fig. 1. Schematic plot of a conventional FFF system comprising carrier delivery fractionation channel, external field force generation and control, detectors and data processing; c: concentration, x: distance to accumulation wall.

the size distribution is usually calculated by using the dynamic signal response of an HPLC-type detector coupled to the channel outlet. In general the characteristic of a derived size distribution (particle number, volume or mass distribution) is therefore determined by the individual sample property detected by the applied detection system. In the analysis of natural colloids with FFF a UV–vis spectrometer is used in most cases [11,15] and the signal obtained originates from true light absorption if macromolecules (as humic acids) are analysed or turbidity from non-absorbing solid (mineral) particles.

1.3. Nephelometric or scattering mode of a fluorescence detector

The signal response in FFF-UV–vis analysis of solid natural particles between ~10 and 1000 nm is related to the turbidity caused by the particles in the optical cell of the photometer. Two different concepts for measuring turbidity can be applied (Fig. 2): light attenuation (turbidimetry, light source, cell and detector are on-axis as in a UV–vis spectrometer) and light scattering (nephelometry, one or more detectors are situated at a

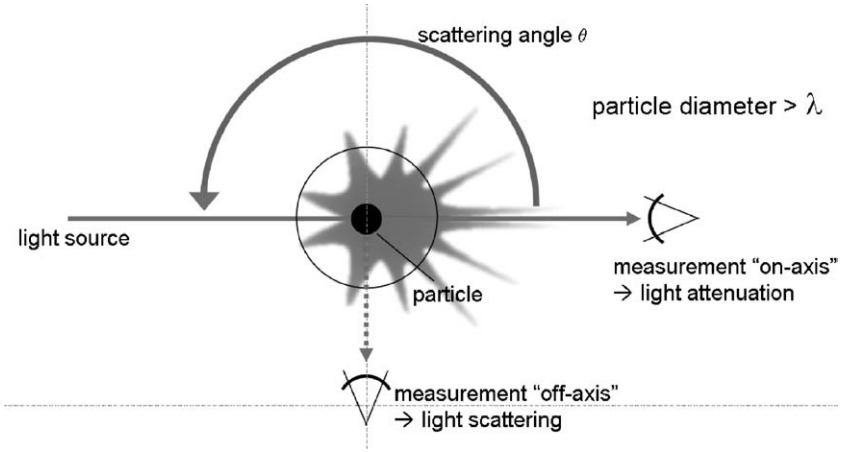

Fig. 2. The turbidity caused by non-absorbing particles in suspension is due to light scattering from the particles. On-axis a light attenuation is observed (classical UV–vis photometer set-up) when the incident light intensity is scattered into directions other than the detector aperture angle. Off-axis a fraction of the scattered light is observed (nephelometry).

certain angle (mostly one at 90°) to the light source-optical cell axis). As long as the particles are not much larger than the wavelength of the incident light both systems are however based on the principles of light scattering. If particles are much smaller than the incident wavelength (λ) light is scattered uniformly into all angles (*Rayleigh scattering*), larger particles but smaller than λ will scatter the light predominantly into the forward direction (*Debye scattering*). If particles are equal or larger than λ the scattering pattern from spherical particles shows distinct minima due to destructive intraparticle interference of the scattered light waves (*Mie* or *Fraunhofer scattering*) as depicted in Fig. 2. Nephelometric turbidity detection in general has the advantage of being less disturbed by substances that truly absorb light. In principle being a light emission technique it may achieve better sensitivity and detection limits compared to absorption techniques. In fact highly sensitive turbidimeters are based on nephelometry and international standard methods for the measurement of turbidity require the use of nephelometric turbidity detectors [17]. The application of those typically batch-cell systems in FFF analysis is prevented by the large internal volumes (∼30 mL) of most commercially available flow-through cells.

In the following the use of an HPLC fluorescence detector, operated in a nephelometric turbidity mode, as main detector in FFF is proposed as addition to, or even as replacement for the UV–vis detector.

The main and critical difference in using a fluorescence spectrometer as a nephelometric turbidity detector compared to normal fluorescence operation is that the excitation wavelength is set identical to the emission wavelength ($\lambda_{ex} = \lambda_{em}$). This basically creates a comparable cheap light scattering detector with one 90° observation angle operating at wavelengths freely selectable within the detectors limitations.

This technique was proposed as an alternative in FFF detection by [16] and by applying general light scattering theory (Rayleigh scattering) also for the successful analysis of molecular weight (M_w) of bovine serum albumin, ribonuclease A and aldolase by [18] in 2000.

1.4. Special concerns on FFF analysis of environmental colloids

For obtaining quantitative results to construct a size distribution from an FFF experiment the detectors response must be related to the particle or macromolecule properties, preferably the eluting particles volume or mass. Particle volume or mass distributions from the detector signals may then be calculated. These prerequisites may be fulfilled when well defined macromolecules are fractionated and detected by light absorption in an UV–vis spectrometer. If the eluting particles or molecules have a constant extinction coefficient ε over size or molecular weight (M_w) or if a function of $\varepsilon(M_w)$ is available, the size distribution obtained is fully quantitative.

However, if solid particles with sizes smaller or comparable to the wavelength of the incident light are fractionated, the attenuation in an UV–vis spectrometer is solely based on light scattering phenomena. Following light scattering theory the signal obtained is no longer dependent on particle mass concentration alone but also on particle size, shape and optical properties as refractive index [19]. Additionally manufacturers attempt to minimize the deterioration of absorption measurements by contributions of light scattering. The response function of such a detector for turbidity measurements is difficult to predict. Several attempts have been published to overcome drawbacks of the in-fact non-quantitative detection with UV–vis spectrometers but are restricted to micrometer size particles [20] or homogeneous samples with certain particle shape [21]. From samples of low heterogeneity (e.g. monodisperse latex beads) the turbidity spectrum from a UV–vis spectrometer can theoretically be used to calculate particle size or correct the response towards quantitative mass concentrations [21,22].

2. Methods and materials

2.1. Samples

The spherical particles used were monodisperse polystyrene latex beads (*Duke Scientific Nanospheres*) in the diameter range from 19 to 1034 nm. Two natural samples A and B containing inorganic natural colloids were obtained by cold water extraction of soil samples. The samples contained (A) 180 and (B) 70 mg/L colloidal particles respectively. Colloid concentration was measured by filtration over 0.02 μm *Anopore* filters (*Whatman*).

2.2. Equipment

The symmetrical Flow-FFF (F-1000) and Sedimentation-FFF (S101) systems were purchased from *FFFractionation* (Salt Lake City, Utah; today *PostNova Analytics*, Germany) equipped with *Hewlett-Packard* HP1100 series quaternary pump, degasser, autosampler and ultraviolet diode array detector (UV DAD) and fluorescence (FLD) detector. To obtain measurements in $\lambda_{ex} = \lambda_{em}$ setting the filter glass on the emission-side PMT tube has to be removed, the "fit spectral range" option in FLD software settings must be disabled and the warning messages must be ignored.

3. Results and discussion

3.1. Spherical and monodisperse particles

To investigate the general properties of signals retrieved in turbidity (UV–vis) and nephelometric mode (FLD) for ideal spherical particles, the response from 14 monodisperse particle size standards between 19 and 1034 nm was determined. Single *Nanosphere* particle size standards at a concentration of 1 mg L^{-1} each and at a carrier flow rate of 1 mL min^{-1} were sequentially injected from the autosampler (5 μL sample volume) into a PTFE tube of 30 cm length and 3 mm i.d. where dispersion caused peak broadening which resulted in a comfortable signal for the following detectors (no FFF fractionation was applied here). The detectors were set at different detection wavelengths between 200 and 750 nm (UV DAD) and 280 and 700 nm (FLD, $\lambda_{ex} = \lambda_{em}$) and runs repeated with the same standard until

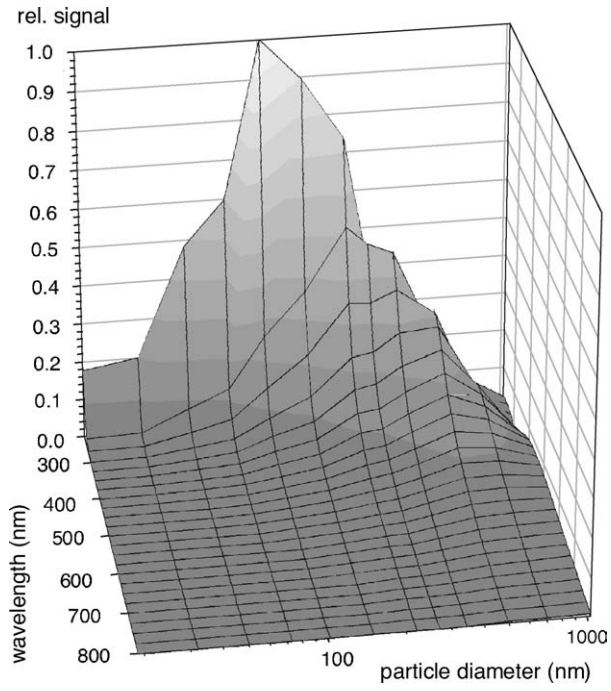

Fig. 3. Relative turbidity signals from UV DAD. Peak area averages of five replicate injections of 5 μL monodisperse *Nanosphere* particle size standards at 1 mg L^{-1}. Values are normalized to the maximum value obtained (Ø 102 nm and λ = 225 nm).

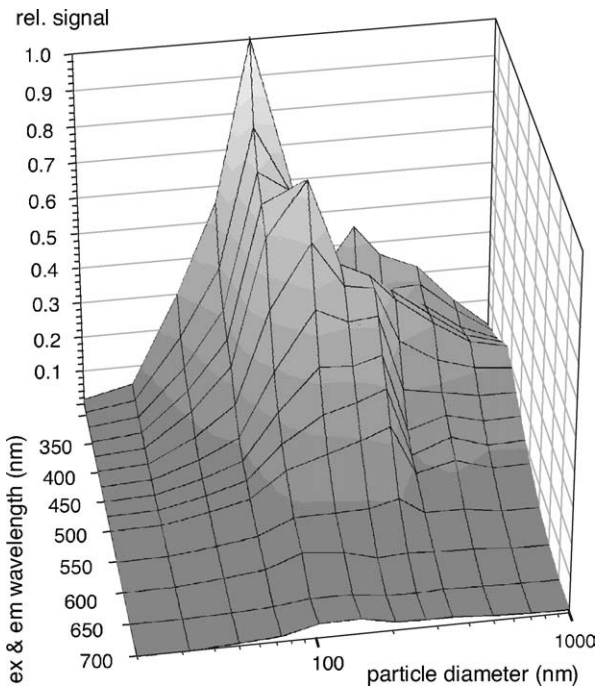

Fig. 4. Relative signals from FLD in nephelometric or scattering mode. Peak area averages of five replicate injections of 5 μL monodisperse *Nanosphere* particle size standards at 1 mg L^{-1}. Values are normalized to the maximum value obtained (Ø 102 nm and $\lambda_{ex=em}$ = 280 nm).

data for all wavelengths were acquired. Fig. 3 shows the results for the UV DAD. For plotting the signal versus particle size and detector wavelength the signal measured was normalized to the maximum overall value obtained.

The 3D diagram in Fig. 3 shows one of the main drawbacks of using a technique as the UV–vis detection for measuring turbidity. The signal obtained at λ = 225 nm is clearly influenced by light absorption from the smaller particles due to the absorption band of the polystyrene, the main constituent of the *Nanospheres* applied. Only at larger wavelengths the signal is a function of wavelength and particle size as already pointed out. If polydisperse dispersions of spherical particles are fractionated by FFF-UV–vis the obtained distribution function will be biased to certain particle sizes depending on the wavelength chosen.

On the contrary, for samples containing natural particles which remain to some extend heterogeneous in size, shape and refractive index even after FFF fractionation, Beckett and Hart [11] found a good correlation between the signal obtained by UV–vis detector and ICP-MS concentrations for main elements which constituted the particles. Similar observations were made throughout our own work and with recent FFF-ICP-MS couplings [14]. This will be discussed in more detail together with data retrieved from natural samples.

The signals obtained in FLD operated in nephelometric mode are shown in Fig. 4. The detector measures the light scattered by the particles at 90° of the incident light (± some unknown angle which is defined by the detector optics). However certain differences to classical light scattering photometers exist. The incident light is not polarized, coherent or monochromatic and while laser light scattering photometers like the *Wyatt Technology "Dawn"* series use a laser as light sources (intense but fixed wavelength) and simple photodiodes as detectors, in the FLD the light source is comparably weak but variable in wavelength and the signal is amplified in a photomultiplier (PMT) on the detection side. For particles with spherical shape, identical refractive index and mass concentration the resulting signal at 90° is a function over size and wavelength. According to light scattering theory and in the limits of the Rayleigh–Gans–Debye approximation [19,23,24] the angular distribution of the excess scattered light intensity given as excess Rayleigh ratio at angle θ $R(\theta)$ for spherical particles may be described by the following equation:

$$R(\theta) = KcMP(\theta) \tag{1}$$

with the experimental constant K

$$K = \frac{4\pi^2 n_0^2}{N_A \lambda_0^4} \left(\frac{dn}{dc}\right)^2 \tag{2}$$

and the particle form factor $P(\theta)$ for homogeneous spheres which describes the intra-particle interference effects:

$$P(\theta) = \frac{9}{qr^6}[\sin qr - qr \cos qr]^2 \tag{3}$$

where the scattering vector q is given as:

$$q = \frac{4\pi n_0}{\lambda_0} \sin\left(\frac{\theta}{2}\right) \tag{4}$$

M is the molecular weight, c the mass concentration, n_0 the refractive index of the solution, dn/dc the refractive index

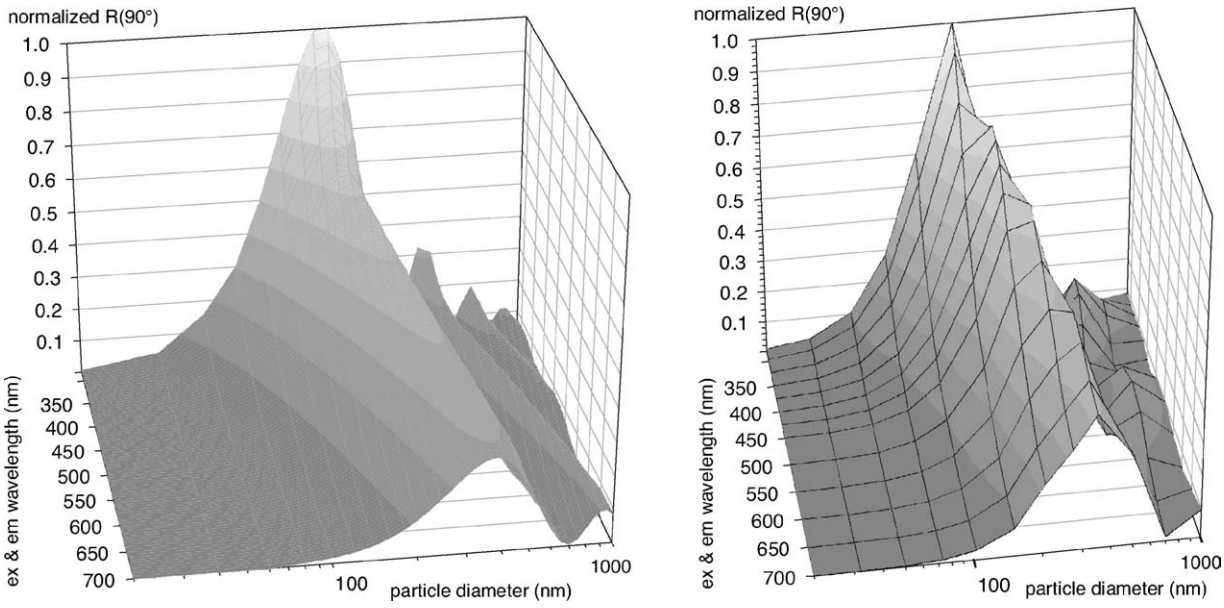

Fig. 5. Relative signal values to be expected for FLD at ∼90° angle (here: average of excess Raleigh ratios R from 82 to 98°) obtained from theory as given in Eqs. (1)–(4) normalized to maximum. Left high resolution plot, right: plot obtained when using particle sizes and wavelengths as applied in experiment. As the particles are identical and relative values are calculated K is reduced to $1/\lambda^4$, c is the constant and M is calculated as d^3 for spheres.

increment and N_A is the Avogadro's number, λ_0 the wavelength of incident light in vacuum and r the particle radius.

Applying these equations to $R(90°)$ results in the relative signal values shown in Fig. 5. For better comparison with experimental data the respective calculated data are given at high-resolution and separately for sizes/wavelengths used in the experiment. In principle the simulation reflects the experimental data fairly well. For wavelengths above ∼400 nm the drop in experimental signal height compared to the simulation must result from effects taking place in the detector which are not covered by the theoretical approach. One reason may be the quantum efficiency of the PMT which is not constant over the whole wavelength spectrum. With some types the efficiency is decreasing logarithmically from ∼400 nm.

For a correct quantitative determination of size distributions from multi-standard or polydisperse samples containing spherical particles in FFF-UV–vis or FFF-FLD a simple and routinely applicable correction function to calculate mass concentration for each slice from obtained signals seems unavailable by now. Respective caution must be applied when reporting quantitative size distributions for samples similar to the described ones.

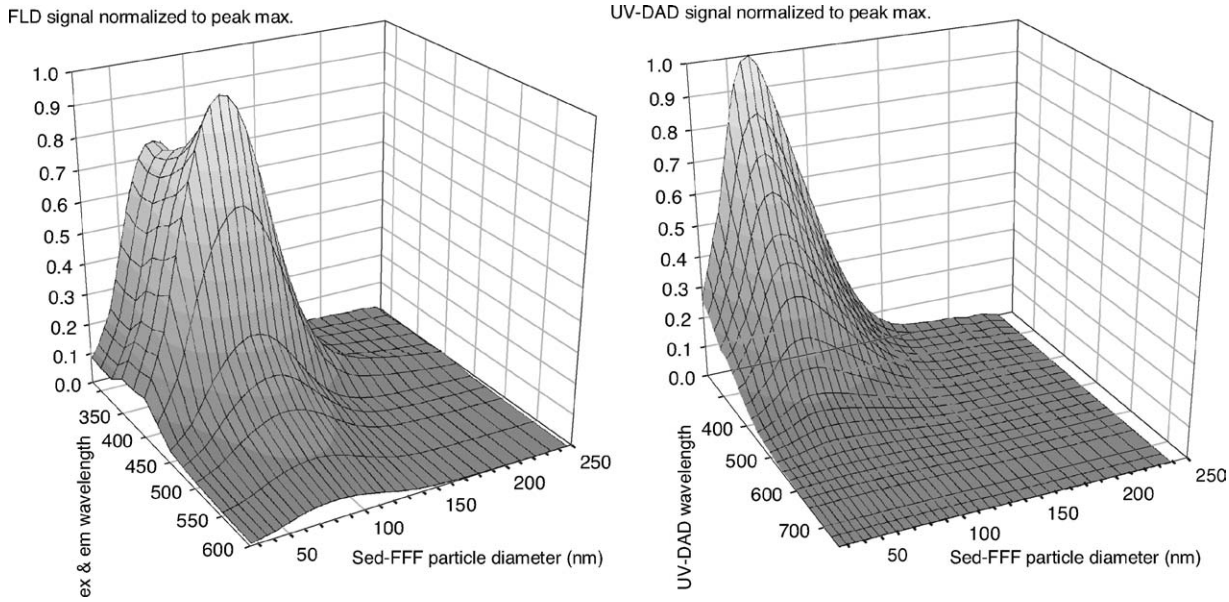

Fig. 6. Three-dimensional plots of peak maximum normalized signal traces from FFF fractionation of a natural colloid dispersion (stabilized cold water soil extract, sample A). Left: FLD response in nephelometric mode, amplification factor pmt = 8, right: UV DAD response with slit width = 20 nm.

3.2. Natural colloidal samples

A rather different picture can be drawn from experimental data obtained from dispersions containing natural colloids. These samples are heterogeneous in several parameters as particle shape, refractive index and internal structure. Even after FFF fractionation a certain heterogeneity will remain in each detected sample slice eluting from the channel. But FFF provides a fractionation (and hence a reduction of heterogeneity) of the bulk sample according to the underlying principles. In the experiment with *Nanosphere* particles the mass concentration of each applied particle size was always constant. With natural colloidal samples a constant concentration over size cannot be provided. Instead two different dispersions of natural colloids were fractionated according to their particle size and results are plotted versus the applied detection wavelength. If the same effect as observed with *Nanosphere* particles applies, the detector signals over particle size as well as the derived size distributions should show a clear dependency from the wavelength applied. This should be visible especially in the shape of the obtained distribution function.

As shown in Fig. 6 there is a clear and not unexpected dependence of peak area and height depending on the applied wavelength, but also no clear change in the shape of the distribution is visible. The maxima vary by some nanometers in particle size and the UV DAD seems to be more sensitive for smaller particles what results in a slight left-shift of the distribution compared to the FLD data. These findings were supported from experiments with FLD detection applying sample B which is essentially broader in its particle size distribution (Fig. 7). Although sample B spans a particle size range comparable to the experiment with *Nanospheres*, there is no strong distortion in the obtained size distribution in dependence of the wavelength used. The measurements at $\lambda_{ex=em}$ = 300, 350 and 700 nm are almost identical varying slightly above the reproducibility of repeated FFF runs (∼5%).

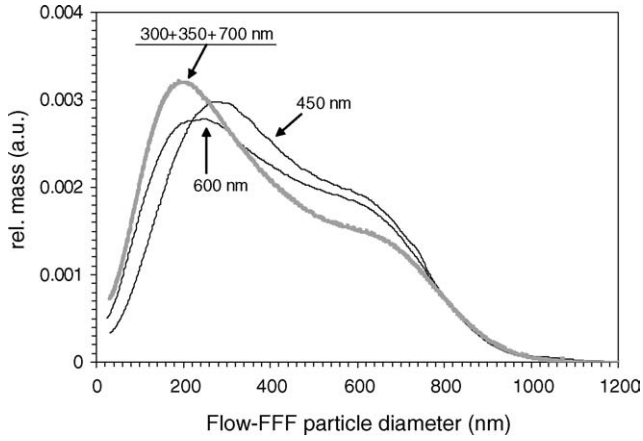

Fig. 7. Size distributions obtained from sample B, a broad distributed natural colloid dispersion in FLD nephelometric mode, amplification pmt = 8, distributions are normalized to area. The respective $\lambda_{ex=em}$ are given in the plot, the bold line represents the average of the distributions obtained with three different wavelengths. By comparison to other sizing techniques the deviations can be attributed as negligible [8].

The results presented for natural colloidal dispersions confirm the findings of Beckett [11,25] that while analysing natural colloids with FFF the signal trace obtained by UV–vis detectors match fairly well the signal trace of main element concentrations as, e.g. Fe measured with ICP-MS.

The data presented show a similar behaviour for the nephelometric detection. Light scattering effects as the intraparticle interference, which becomes dominant when particles are about or larger than the wavelength, seem to play a minor role compared to spherical particle standards. It must be stated that of course the principles of Rayleigh scattering also remain true with the natural colloid dispersions tested here. The d^6-dependence of scattered light at constant particle number concentration (d: particle diameter) causes a strong decline of the *signal* to *particle mass concentration* ratio if the particle diameter is much smaller

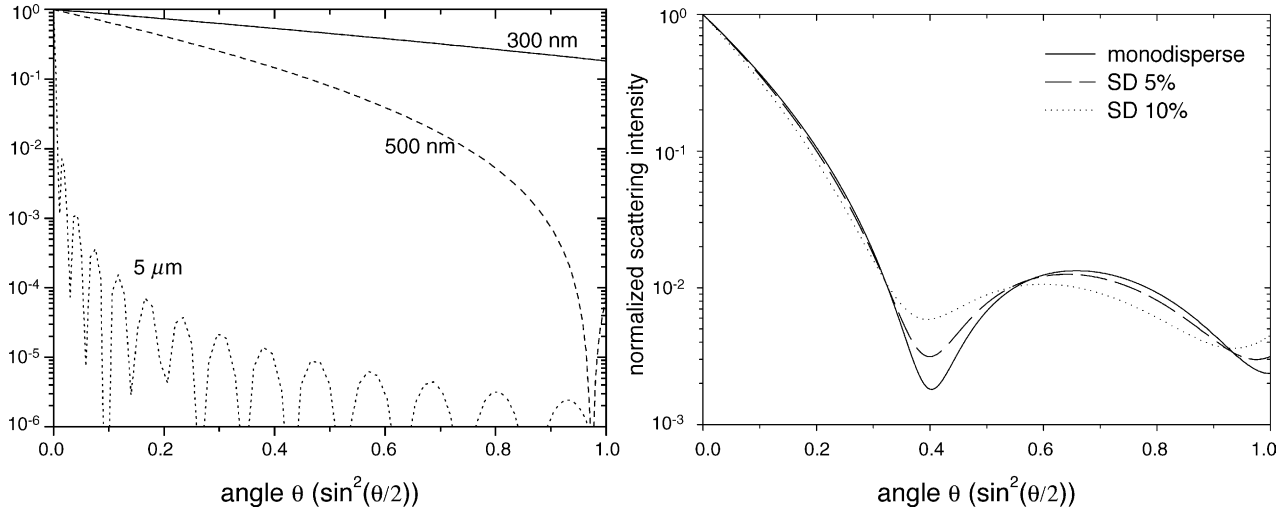

Fig. 8. Left: simulation of the angular distribution of light scattering intensity by means of $P(\theta)$ using (3), incident light λ = 690 nm, monodisperse homogeneous spheres of 300, 500 and 5000 nm diameter. Right: applying *Mie* theory: homogenous spheres of 700 nm as monodisperse and two polydisperse samples (calculated in *Mie-Plot V3.4.05* Software).

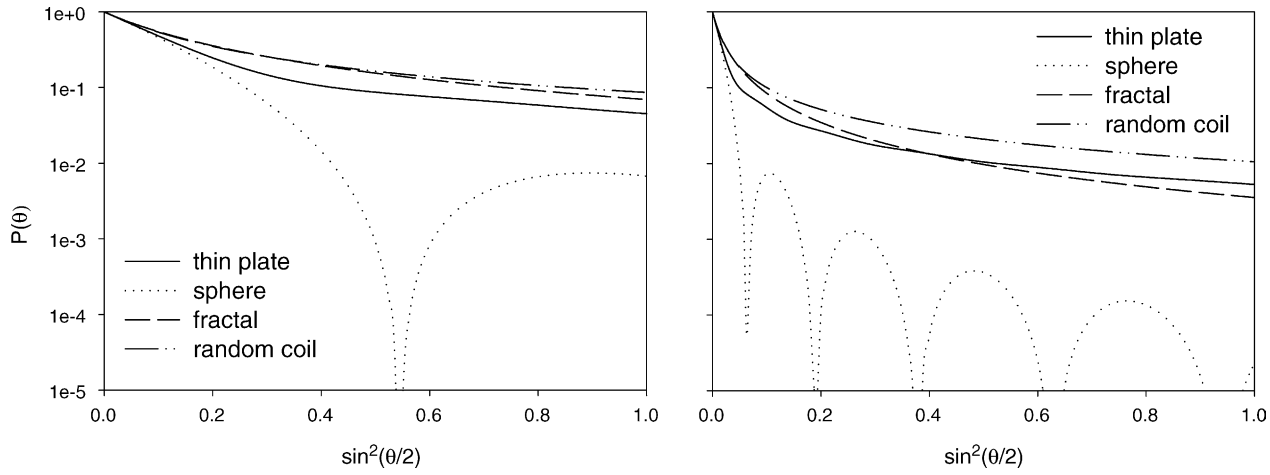

Fig. 9. Left: simulation of the angular distribution of light scattering intensity by means of different geometrical models for the form factor $P(\theta)$, incident light $\lambda = 320$ nm, thin plate main axis radius and particle $r_g = 120$ nm (corresponding sphere $r = 158$ nm what equals $\sim\lambda/2$); right: same situation, but particle $r_g = 350$ nm (corresponding sphere $r = 452$ nm); here fractal dimension df was set to 3.

than the applied wavelength. Therefore with wavelengths set to about $\lambda = 300$ nm particles <Ø 30 nm will not be represented correctly in the derived size distributions. This effect seems to be stronger for the FLD than for the UV–vis detection. This can be seen in the shift to smaller particles of the UV–vis signals in Fig. 6, the reason may be a higher sensitivity for the smaller particles compared to FLD detection.

The reason for the essentially different detector response for monodisperse spherical and natural arbitrary shaped particles can be explained when the differences in the particle form factor $P(\theta)$ and the remaining polydispersity after fractionation of natural particles are considered. If a broad distributed sample is fractionated the particles present in the detector cell will not be completely monodisperse due to band broadening effects in FFF. Sharp minima as produced from $P(\theta)$ of homogenous spherical particles will be smoothed as soon as the size distribution is not longer monodisperse. This effect is shown in Fig. 8 for different monodisperse spherical particles and a polydisperse sample based on (3).

Moreover the particle form factor $P(\theta)$ for particle geometries similar to those occurring in natural samples does not show those deep minima as observed from spherical particles of larger diameter (Fig. 9). If the shape of the particles is assumed as, e.g. a fractal aggregate with a mass fractal dimension of 2 the plot in Fig. 5 changes into a much more simple situation which is shown in Fig. 10. The $P(\theta)$ of fractal aggregates is given as [26]

$$P(\theta) = \left(1 + \frac{2(qr_g)^2}{3\mathrm{df}}\right)^{-(\mathrm{df}/2)} \quad (5)$$

with r_g the root mean square radius of the aggregate [23] and df the mass fractal dimension (between 1 and 3). However, the actual shape of the particles in the detection cell may be spherical, ellipsoidal, "plate-like" as clay particles or may have the appearance of fractal aggregates and the actual assembly of different particle shapes in the detector cell cannot be assessed. Results from natural samples and the fact that $P(\theta)$ of thin plates behaves quite similar to the results shown in Fig. 10 allows to conclude that presented results underpin the usability of both detector systems as a semi-quantitative detection system after FFF fractionation of non-absorbing natural particles.

To compare the analytical performance of the turbidity measurements by FLD and UV–vis the signal to noise (S/N) ratios were obtained from the *Nanosphere* experiments. Fig. 11 shows that the UV–vis detection has an advantage with smaller wavelengths below 300 nm while FLD shows a much less dependence of S/N ratios from the applied wavelength and will be

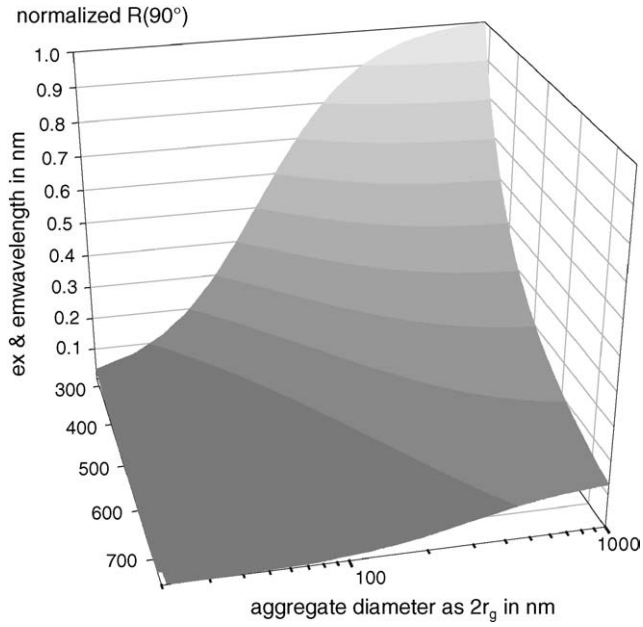

Fig. 10. Relative signal values for fractal aggregates with a mass fractal dimension of 2 (spheres: 3) to be expected for FLD at 90° angle obtained from theory as given in Eqs. (1), (2), (4) and (5) normalized to maximum. As the particles are identical and relative values are calculated K is reduced to $1/\lambda^4$, c is the constant and M is calculated as d^2 for thin plates.

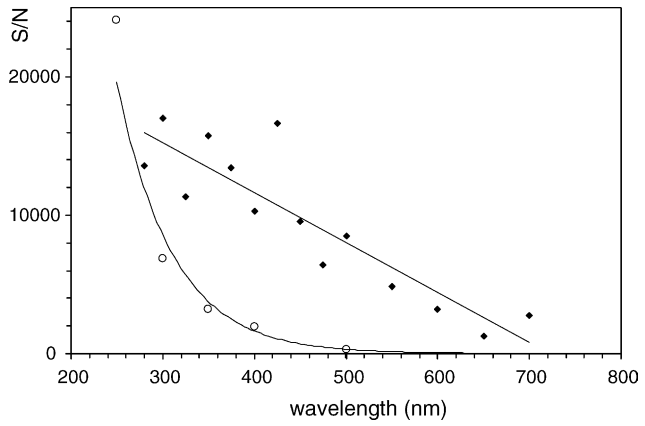

Fig. 11. Signal-to-noise ratio calculated from *Nanosphere* experiments (Ø 102 nm) as a function of incident light wavelength. Standard deviation of 5 min baseline recordings were used as noise level. FLD: pmt = 8; UV DAD: slit width 20 nm.

comparable or better than UV–vis detection from $\lambda > 300$ nm. The steep increase of S/N ratios of the UV–vis to smaller λ was first accounted to true light absorption of the polystyrene nanospheres resulting in relatively high signals, but also the values obtained from the natural samples show a clear advantage of the UV–vis at small wavelengths.

4. Conclusions

The application of UV–vis detectors to generate particle size distributions after FFF fractionation has to be done with serious caution and regard to the type and characteristics of samples fractionated. Regions of relative safety are existent where light scattering effects usually observed from monodisperse spherical particles affect the signal to a lesser extent, especially at large wavelengths. However, this comes to a certain cost: a reasonable loss of sensitivity and S/N ratios at larger wavelengths. The approaches to correct UV–vis data from spectral information as worked out by [20,21] may lead to a fundamental change of this conclusion but are yet not easily available or even routinely applied. The commonly accepted fact under practitioners of FFF for the fractionation of natural colloidal dispersions that the UV–vis is a quantitative signal for the apparent particle mass concentration could be underpinned by experimental data formerly not available. However, for very small particles this common sense does not hold true and the detection system must be regarded as semi-quantitative and limited to certain size-ranges.

The applicability of a HPLC-type fluorescence detector as a nephelometric turbidity sensor in a detection mode which is usually not supported by the manufacturer could be proven and the same conclusions from experiments with UV–vis detectors apply. The main advantage is a better signal to noise ratio at larger wavelengths and the much lesser sensitivity against truly light absorbing substances like humic acids. First attempts to use light scattering theory for modelling the resulting signals obtained from spherical particles show that correction functions can be developed similar to the approaches in UV–vis detection. For the further development of a theoretical treatment of observed effects the special characteristics of the optical system must be taken into account.

Typically in environmental Sed FFF fractionation particles from ~20 to 800 nm in diameter are fractionated while small particles and light absorbing macromolecules elute in the void peak thereby disturbing the main signal peak. It seems advisable to use FLD in nephelometric mode in this application as a concentration detector.

The spectral abilities of the FLD detector used in this study (data not shown) enables the parallel detection of turbidity and (if the correct wavelength is chosen) of fluorescence signals on the other three emission wavelengths available or even in full spectral mode.

The application of a nephelometric turbidity measurement using HPLC-fluorescence detectors is not restricted to FFF analysis. This technique may also apply elsewhere where small flow rates or small volumes have to be investigated for turbidity.

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