

4 Laser diffractometry – investigations on the method and on the performance of the LS 230

4.1 Theoretical background

Particle size characterisation by laser diffractometry is a frequently used technique. Particles of sizes from 10nm up to 2000 μ m can be analysed by this technique. The broad measuring range, obtained by only one measurement, as well as fast and reproducible measurements are the pronounced advantages of laser diffractometers.

The principle of laser diffractometry, also called static light scattering, is the detection of diffracted light. In contrast to dynamic light scattering technologies, here any motions of the particles are non relevant, as the interaction of light with the particles is instantaneous. The theory of diffraction is based on the wave nature of light. When a coherent light beam strikes the border of a particle it gets illuminated and a specific interference pattern, called Fraunhofer diffraction rings, is generated. Such an obtained pattern of diffraction can be nicely compared with a stone vertically dropped into water. If the stone hits the water, it produces lobes around it, similar to the Fraunhofer diffraction rings. The first lobe is highest and the intensity of the maxima decreases with an increase of the distance from the centre. However, imagine a large stone dropping into water; one will see a big splash with very narrow lobes occurring next to the stone. Now imagine a very small stone, when it drops into water, here one only sees very little lobes. Also the distance from the stone to the first lobe is much broader. These two characteristics are important for laser diffractometry. The intensity of diffracted light decreases linear with the size of the particle. Similar to the stone, also the first lobe of a diffraction pattern obtained incorporates most of the energy of the diffracted light, which is 75% of the total energy. The angle of scatter and the position of the lob's minima and maxima are described by the Airy formula. From this it can be calculated, that the angle of scatter increases logarithmically with a decrease in particle size. The minimum of the first lobe is at $1,22\lambda / 2d$, where λ is the wavelength of the incident light source and d is the diameter of the particle (Horiba-Instruments 2004).

Incident light causes not only diffraction but also other scattering phenomena, e.g. reflection, refraction and absorption followed by re-radiation. Reflection is the abrupt change in direction of a wave front at an interface between two dissimilar media, so that the wave front returns into the medium from which it originated. Refraction is the bending of light due to a change in its speed. Absorption is the transfer of energy carried by light waves to particles in matter. This energy is reradiated as heat or light.

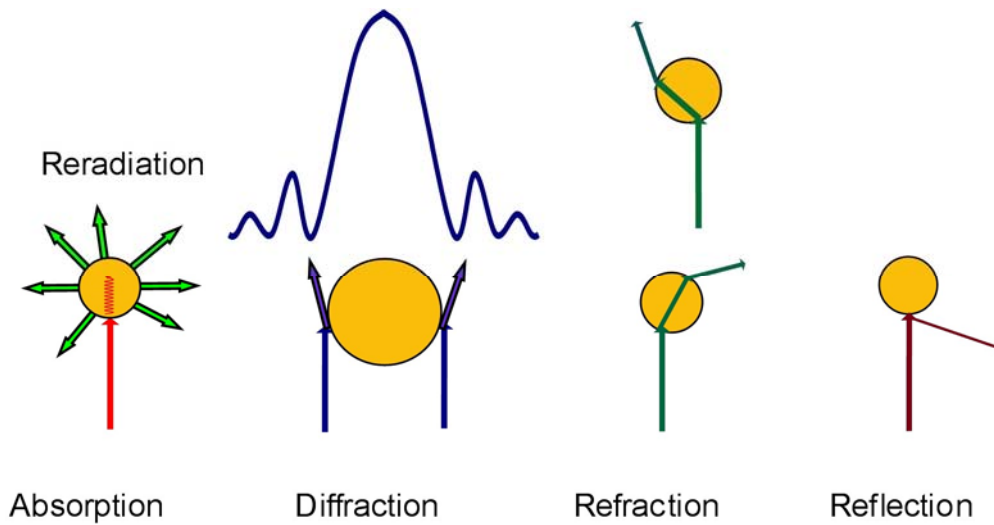


Figure 4-1: Overview of scattering phenomena which occur if a particle is illuminated with light: absorption, diffraction, refraction, reflection

Diffraction is the bending, spreading and interference of waves when they meet an obstruction or gap that is larger in size than the wavelength of the wave. Figure 4-1 shows the different phenomena in a simplified form. Therefore the intensity of scattered light is the sum of all this phenomena ($\Sigma \text{ intensity}_{\text{light}} = \text{reflection} + \text{refraction} + \text{absorption} + \text{reradiation} + \text{diffraction}$). As all these phenomena occur if a particle is illuminated with light and need to be taken into account for LD analysis therefore.

The theory behind this is the Mie theory, which was invented by Gustav Mie in 1908. It describes all effects from incident light on a spherical particle. The Mie theory themselves is very complex, but in a simplified form (see Formula 4-1) it is visible that is consistent out of three terms. The first term describes Fraunhofer scattering, the second part the Mie scattering and the third term describes Rayleigh scattering. As one can see from the formula, the second and the third part become very small if particles become very large. Therefore they can be ignored for analysis in those cases. If particles become very small the first and the second term become so small, that they can be ignored for analysis. The first case explained is called Fraunhofer Scattering, the second case is known as Rayleigh scattering.

Formula 4-1: Mie theory in a simplified and schematic form after (Horiba-Instruments 2004)

$$I(w) = E \left\{ \underbrace{k^2 A^4 [J_1]^2 W^1}_{\text{Fraunhofer Term}} + [K_1 W]^1 + [K_2 W]^3 + [K_3 W]^5 + \underbrace{k^4 A^6 (m-1)^2 W^6 / 8\pi^2}_{\text{Rayleigh Term}} \right\}$$

- I= intensity of scattered light**
- E= flux per unit area of incident light**
- k/K= constants**
- A= particle radius**
- J1= first order Bessel function of first kind**
- W= angle of scatter**
- m= complex refractive index (sum of imaginary and real refractive index)**

The differences of the different scattering patterns are shortly explained here. Particles showing Fraunhofer scattering have a very strong forward scattering. Intensity of scattered light is very intense. Smaller particles with Mie scattering show a decreased forward scattering and a decreased intensity. The intensity of the light scattered decreases linear with a decrease in particle size (see Figure 4-2).

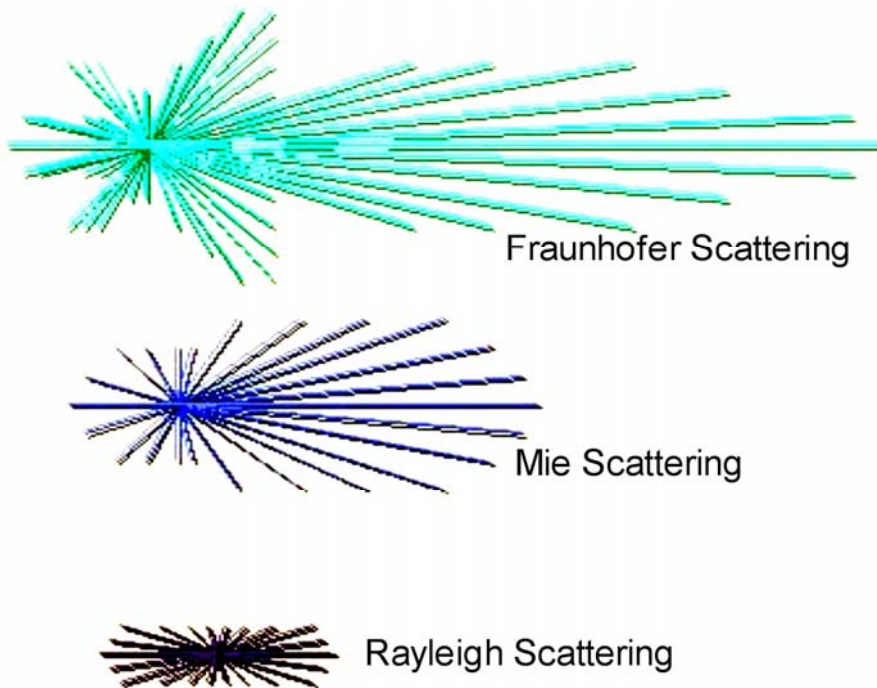


Figure 4-2: Patterns of scattering Fraunhofer, Mie und Rayleigh scattering

As one can see from Formula 4-1, the forward scattering of large particles is such intense, that influences as intensity from refracted light and absorbed light become so small, that they can be ignored for particle sizes analysis by static light scattering techniques. This corresponds to Fraunhofer analysis. If particles become smaller the forward scattering decreases and intensities of light from refracted and absorbed light need to be taken into account. This corresponds to Mie analysis. Particles showing Rayleigh scattering characteristic have a very symmetric scattering pattern, when forward and backwards scattered light intensity is compared. Those patterns have no angular information anymore, therefore particles with Rayleigh characteristics cannot be analysed by laser diffractometry. It is important to notice, that scattering patterns obtained from particles are dependent on the ratio of wavelength of the incident light to the particle size. Particles, much larger ($>4x$) than the wavelength, show Fraunhofer scattering. Particles with a size around the wave length show Mie scattering. Particles, much smaller ($<10x$) than the wavelength used, show Rayleigh scattering. Table 4-1 gives an overview which particle sizes correspond to the explained scattering models in case of using an LS 230.

Table 4-1: Overview of scattering patterns and corresponding particle sizes obtained when using the LS 230 with a helium neon laser having a wavelength of 750nm

scattering pattern	mode of analysis	LS 230
Rayleigh	---	$<38\text{nm}$
*	Mie mit PIDS*	38-120nm
Mie	Mie	$>120\text{nm}$
Fraunhofer	Fraunhofer	$>3,75\mu\text{m}$

* see Chapter 4.3.1

Particles smaller than 38nm show Rayleigh scattering and can not be analysed by the LS 230. Particles from 38-120nm can only be analysed by applying additional techniques, as the angles of scattering are such big, that diffracted light would be detected at the upper end of the detector elements. Also the intensity would be very low, making a differentiation of particle sizes in this range impossible. The wavelengths of lasers used in instruments of different manufactures vary, also the techniques used to detect smaller particles. Therefore also the sizes and the corresponding scattering patterns viewed in Table 4-1 are different for each instrument used.

4.2 Overview of laser diffractometers on the market

The most important manufactures of laser diffractometers are Malvern Instruments, Horiba Instruments, Cilas, Beckman-Coulter and Sympatec. Fritsch, more known for manufacturing mills, also manufactures laser diffractometers. The influence of Fritsch instruments in the international market is small; it was included in this chapter, because of its interesting technique.

The first laser diffractometer was brought into the market from the company Cilas in the early 80ties. The measurement range was only from 400nm–1000 μ m. Since than the capacity of personal computers as well the accessibility of lasers of different wavelengths has increased constantly. The introduction of nanotechnologies has brought up the interest to develop instruments with the capacity for analysing even particles in the lower submicron range by this technique. The request of the customers was fulfilled. Today instruments are available on the market measuring particle sizes ranging from only 20nm up to 8750 μ m.

In general all instruments have the same construction, which is shown in Figure 4-3. It consists of a laser as the light source and the optical system. This consists of lenses to widen the laser beam to ensure the illumination of the complete sample. The sample is added to the sample cell and the Fourier lens ensures the bundling of the diffracted light from many identically sized particles in different positions to the detection rings on the detector. Independent on the particle position, the patterns of differently located particles match. The detected intensities, which are the raw data, are transferred to a PC and analysed by the software of the PC.

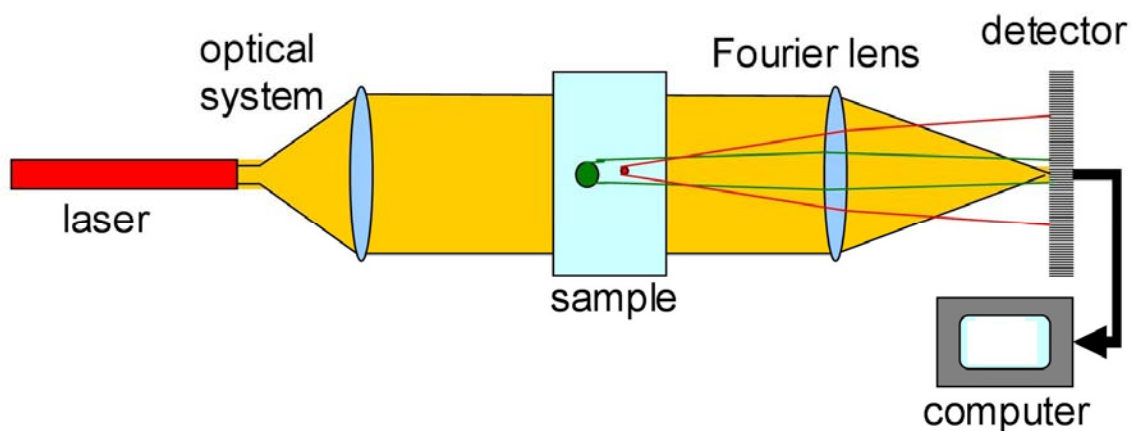


Figure 4-3: Schematic set up of a laser diffractometer (modified with permission after (Müller 1996))

Differences of the instruments are the wavelength of the laser used and the additional techniques for the detection of smaller particles, which cannot be detected by simple laser diffractometry. Due to intellectual property rights each method is different from the others. The basic principle is always the aim to obtain more detailed information about the scattering patterns of the particles. Additional information can be obtained by the comparison of forward scattered intensities and backwards scattered intensities. Also the comparison of intensities at different wavelengths is capable to obtain those required data. This is possible, because the pattern created strongly depends on the ratio of particle size and the wave length of the light source used. Therefore in most of the instruments more than one light source is used, to compare scattering patterns created under different wavelengths. Also intensities are not only measured in forward direction, but also in other angles, which vary from manufacturer to manufacturer. A short overview of manufactures and instruments is given in Table 4-2.

Table 4-2: Overview of manufactures and latest instruments on the market

Manufacturer	Name of Instrument Type	range of measurement	Wave length of laser used	additional technique	approximate price
Cilas	Cilas 1180	0.04-2500 μm	800nm	2nd Laser (632.8nm) at 45°	55.000€
Sympathec	Helos BF/Vario	0.1 μm -8750 μm	632.8nm	shift of laser	55.000€
Malvern	Mastersizer 2000	0.02-2000 μm	632.8nm	2 nd Laser 405nm	55.000€
Fritsch	Analysette 22“NanoTec”	0.1-1000 μm	632.8nm	convergent laser beam, 2nd Laser (632,8nm) measurement of back scattering)	55.000€
Beckman-Coulter	LS 13320	0.04-2000 μm	780nm	Polarisation intensity differential scattering (PIDS)	55.000€
Horiba	LA-920	0.02-2000 μm	632.8nm	tungsten lamp at 405nm, wide angle analysis 0-150°	55.000€

4.3 Measuring principle of the LS 230

The LS 230 is the measurement instrument type prior to the previously launched LS 13320. It is a laser diffractometer with a measuring range from 40nm-2000 μ m, manufactured from Beckman-Coulter, Krefeld, Germany. The light source used is a helium neon laser with a wavelength of 750nm. In general the instrument corresponds to the construction shown in Figure 4-3. However, the measurement range from 40-400nm is only approached by applying the so called PIDS (Polarisation Intensity Differential Scattering). This additional technique enables a more accurate analysis of small particles in the submicron range, as well as the extension of the measuring range down to just 40nm.

4.3.1 PIDS

PIDS in principle measures the change of scattered light intensities of the sample under different wavelengths and angles. The technology was invented by Bott and Hard in 1990 (Bott and Hart 1990). PIDS relies upon the transverse nature of light i.e. it consists of a magnetic vector (M) and an electric vector (E) at a ninety degrees angle (Figure 4-4). If the electric vector is 'up and down' the light is said to be vertically polarised, whereas it is horizontally polarised if the electric vector is 'front and back'. If a particle is irradiated with light of a given polarised wavelength, the oscillating electric field establishes a dipole, or oscillation of the electrons in the particle. These established oscillating dipoles in the particles radiate light in all directions *except* that of the irradiating light source. The radiated light corresponds to the scattering patterns described in 4.1., which are dependent on the particle size in ratio to the incident wavelength. PIDS takes advantage of this phenomenon. The sample is irradiated with first vertical and then horizontal polarised light. The scattered or radiated light from the sample is measured over a range of angles, to record the resulting scattering pattern. The PIDS detectors are placed in a plane at ninety degrees to the sample cell, dependent on the type of polarisation the scattered light derived from the oscillating dipoles within the particle will be detected (horizontal polarisation of incident light) but are not detected if vertically polarised light is irradiated (Ley 1998). Light intensities measured under the influence of horizontally polarised light are only derived from

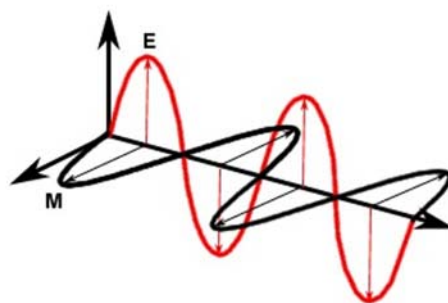


Figure 4-4: Schematic drawing of a light wave, having a magnetic vector (M) and an electric vector (E) at ninety degrees located to each other

other scattering phenomena (e.g. reflection, refraction, diffraction, absorption). By analysing only the differences between the horizontally and the vertically radiated light intensities recorded by the PIDS detectors, the scattering pattern solely based on irradiated light is obtained. Light intensities (yielding the scattering patterns) are obtained at six different angles. Low angles correspond to light intensities of forward scattered light, whereas the larger angles correspond to backward scattered light (see 4.1.). The variation of wavelength changes the ratio of particle size to wave length, and the scattering pattern obtained therefore. PIDS uses wave lengths of 450nm, 600nm and 900 nm. For example, a particle having a size of 400nm would show Mie scattering behaviour at a wavelength of 450nm, at 600nm the intensity of forward scattered light would be decreased and is even more decreased at an applied wavelength of 900nm. In contrast, scattering patterns of larger particles are not much influenced by changes in wavelengths, as Fraunhofer scattering is obtained for every wavelength applied. Therefore, the greater the variation of detected intensities between the different wavelengths the smaller are the particles within the sample analysed. Figure 4-5 shows the particle size distribution (A) and Figure 4-6 shows the corresponding PIDS data (B) for small sized lattices and for a coarse drug powder (cyclosporine). In graphic B three columns are visible. The columns from left to the right correspond to the light intensities recorded for 450nm, 600nm and 900nm. Each column consists of six smaller columns itself, which correspond to the angles of record (60-146, from left to right). All intensities shown give the differences between vertically and horizontally irradiated light by the particles (Detector V-H). It clearly shows that the scattering intensities of the small particles are strongly influenced by the change in wave length, whereas the intensities of large particles e.g. cyclosporine powder are not or very little influenced.

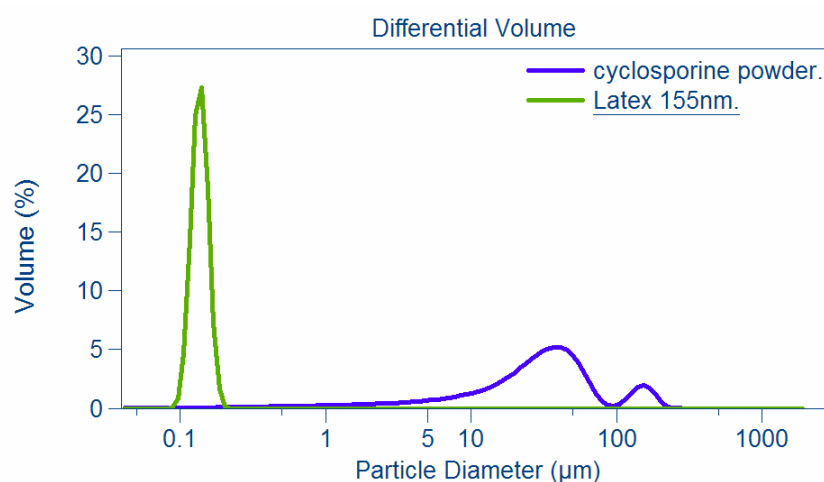


Figure 4-5: Particle size distribution of latex particles 155nm and coarse cyclosporine powder (A)

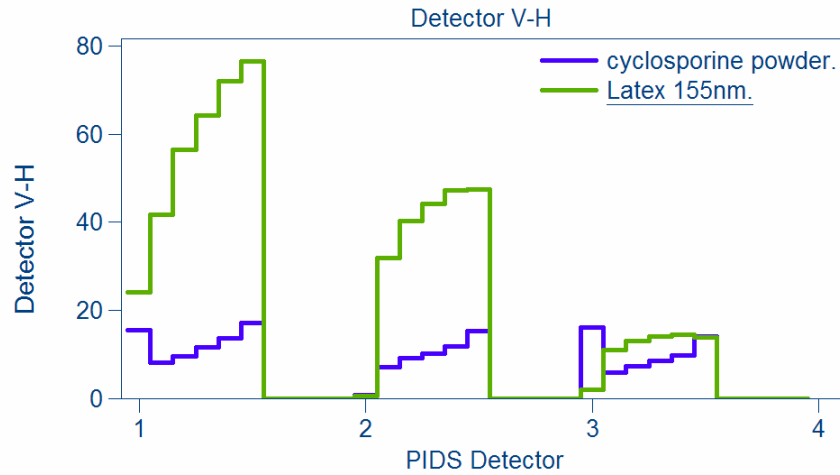


Figure 4-6: PIDS intensities for latex particles and cyclosporine powder measured at 450nm, 600nm and 900nm (from left to right) (B)

Figure 4-7 shows the set up of the PIDS. It consist only of a light source, a filter wheel, the sample cell and the PIDS detectors located in 90° position to the sample cell.

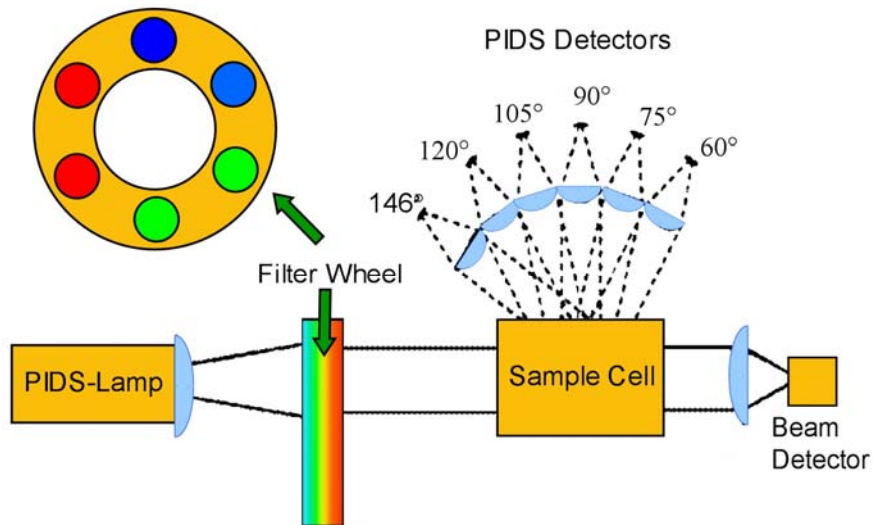


Figure 4-7: Schematic set up of PIDS after (Bott and Hart 1990)

The filter wheel consists of six pin holes, two for each wavelength, one for horizontal polarisation and the second for vertical polarisation. The PIDS intensities obtained are directly incorporated into the standard algorithm from the intensity vs. scattering angle data from the primary laser, giving a continuous size distribution.

4.4 Aim of study

Modern laser diffractometers are combined instruments where not only the diffraction pattern is detected. Also additional information from scattered light of the sample is obtained in respect to its dependency on angle and wave length. Additional techniques are required for the extension of the measurement range down to submicron particles. Those techniques were invented in order to fulfil customer's expectations for fast and simple particle size analysis over a very broad measurement range. Therefore nowadays two independent measurement methods are performed in one single measurement. The obtained raw data of the measurements are then combined by the software to only one analysis result, incorrectly named the result of a static light measurement. Each method includes hazards of errors. The combination of two technologies in one instrument therefore doubles the hazard of systematic errors and of miss usage. Information from literature is very rarely to obtain. The aim of this work was to investigate the performance of the LS 230 in order to identify the advantages and drawbacks, limitations, the hazards of errors and miss usage of the technique. Also instruments from other manufacturers were tested to some extent for a comparison of the performance. From the obtained results new guidelines for an optimised particle size analysis by the LS 230 were established. Also general suggestions for the improvement of particle size analysers are given.

4.5 Nailing test

A test for investigating the sensitivity of an instrument is called nailing test. The name of the test arrived from the slang to nail something, here the performance of an instrument. The aim is to control the accuracy of a method as well as the efficiency for the detection of even small particle fractions within a polydisperse system. In the test systems with a known particles size and size distribution, are analysed. Afterwards the obtained results are compared with the known data.

The LS 230 with the patented PIDS technology is sold as a high dissolution instrument. It is promised by the manufacturer to dissolve even complex multi modal particle size distributions in the submicron range. For demonstration of the instrument a latex dispersion, also manufactured by Beckman-Coulter is used. The analysis in the LS 230 leads to a trimodal distribution of the dispersion in the submicron range and cannot be analysed by other instruments. Therefore the combination of a unique technique and the use of the Beckman-Coulter dispersion were used to convince many customers in the last few years.

However the use of an instrument should not be proved by analysing a sample especially optimised for the particular instrument. It is more useful to investigate the performance by analysing samples which correspond to systems thought to be analysed in the future by the instrument. In the workgroup of R.H. Muller mostly nanosuspensions and solid lipid nanoparticles are analysed. Mean particle sizes are ranging from approximately 100-1000nm. All systems are produced through high pressure homogenisation. The particle size obtained, as well as the width of the distribution depends on the amount of cycles performed by high pressure homogenisation. The aim of a production is to obtain a homogeneous dispersion with a narrow particle size distribution. If too less cycles are performed some large particles, which could escape from the homogenisation process, can be left in the dispersion. Also the mean size can be too large and the size distribution too broad. All those facts cause instability of the systems produced. In some of the cases too many cycles will lead to instability of the systems, as too much energy was applied to the system, which can cause agglomeration or coalescence. Long time storage of the systems can lead to crystal growth, agglomeration and re-crystallisation in case of the drug nanocrystals, leading to an increase of the mean particle size and the appearance of a second particle fractions of some large crystals ranging typically from 5-20 μ m. Solid lipid nanoparticles can agglomerate as well, but here the main hazard is the repulsion of incorporated drug, leading to new particle fractions with particle sizes ranging from 2-200 μ m. Therefore a sensitive instrument is required, which can reliably detect multi modal particle size distributions, as well as changes in those systems.

Three different polydisperse latex dispersions were prepared and analysed (Table 4-3). The aim was to investigate the accuracy and the reliability of the LS 230. The mixtures were prepared in order to simulate dispersions with particle size distributions similar to those expected for nanosuspensions or SLN/NLC. Mixture A represents a system with four different particle sizes (102nm, 404nm, 845nm and 2875nm in a ratio of 18.2:18.2:45.5:18.2w%). Such a system could be obtained for a nanosuspension. The bulk population corresponds to 845nm. Two smaller fractions (102nm, 404nm) represent particles, which could be further diminished. The fourth population (2875nm) could occur if some particles remained unhomogenised. This distribution is thought to be the general case for nanosuspensions. Polydisperse systems are obtained as the diminution process and the following particle size are dependent on the crystal structure of the particles. The more imperfections are within the single crystal, the smaller is the size after the high pressure homogenisation. The number of imperfections varies for each particle homogenised and therefore the resulting size after the homogenisation process. It is also possible that some particles escape from the homogenisation process, those particles

remain in the final nanosuspension as a larger particle population. In order to investigate the limit of the LS 230 also less complex mixtures were produced (mix B and mix C). Mix B simulates a trimodal dispersion with a main population of 404 nm, some particles with a size of 845nm and a population of larger particles, similar in volume to the population of 404nm. Such a distribution can be expected for nanosuspensions only homogenised a few cycles (e.g. 5 cycles at 1500bar). Mix C is bimodal and simulates a SLN or NLC dispersion with a main particle size of 200nm. The second fraction was added to simulate some larger particles (e.g. aggregates or repulsed drug).

All samples were measured three times, by keeping the measuring conditions constant. From the theory, described in the introduction of the chapter, it is known, that particles smaller than 3.75µm should be analysed by the Mie mode. However, the Beckman-Coulter mixture is analysed by the Fraunhofer mode when the instrument is demonstrated. Also the hand book of the LS 230 describes the Fraunhofer mode as the appropriate analysis mode for each measurement. It is only mentioned, that using the Mie mode can lead to slightly more accurate results (see page 101 (Beckman-Coulter 1994)). Therefore all data obtained were analysed in Fraunhofer and Mie mode, the optical parameters used in Mie mode were 1.6 for the real refractive index and 0 for the imaginary refractive index.

In addition all measurements were simulated by using different optical parameters (see Table 4-5) in order to investigate the influence of the refractive indices on particle size and size distribution. The following systems were analysed.

Table 4-3: Composition of the latex dispersions analysed, standard particles were mixed to obtain multimodal distributions

Latex mixture	particle sizes of standards used in µm	volume of added standard* in µl	corresponding volume of added particles in µl	volume of particle population in ratio to the total volume of particles in w%	number of particles** in the mixture
Mix A tetramodal	0.102	8	0.08	18.2	1427
	0.404	8	0.08	18.2	360
	0.845	20	0.2	45.5	431
	2.875	8	0.08	18.2	51
	total	44	0.44	100.0	2268
Mix B trimodal	0.404	8	0.08	40.0	360
	0.845	4***	0.04	20.0	86
	2.875	8	0.08	40.0	51
	total	20	0.2	100.0	497
Mix C bimodal	0.204	20	0.2	94.3	1783
	0.845	1.2****	0.012	5.7	26
	total	21.2	0.212	100.0	1809

*all standards had a particle concentration of 1w%,

**density of latex 1.05g/cm³

*** =20µl, diluted 1:5

**** =6µl, diluted 1:5

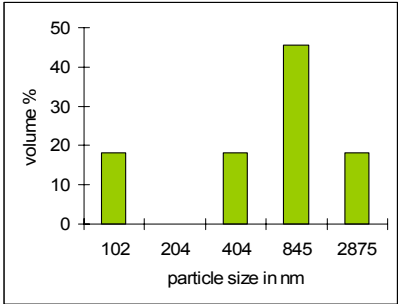
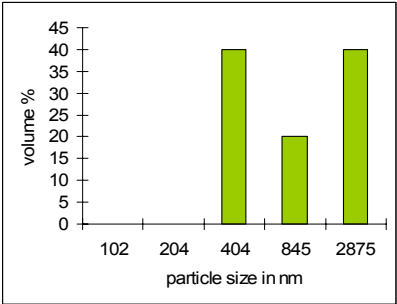
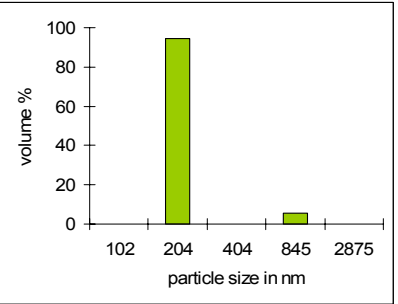
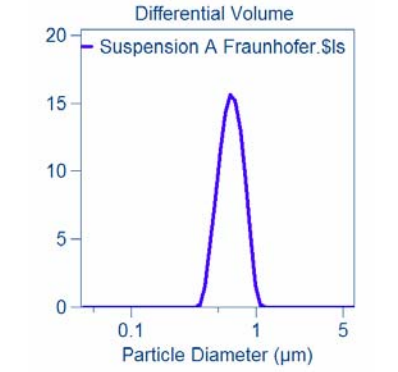
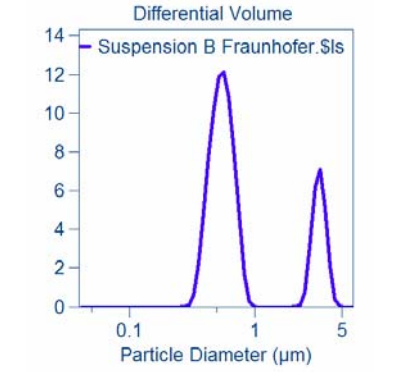
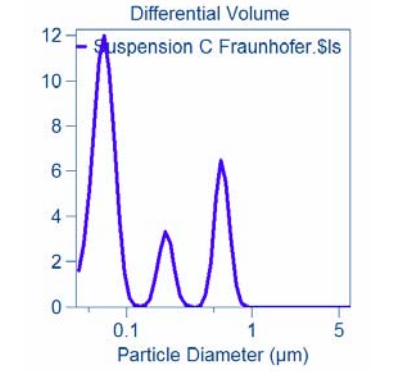
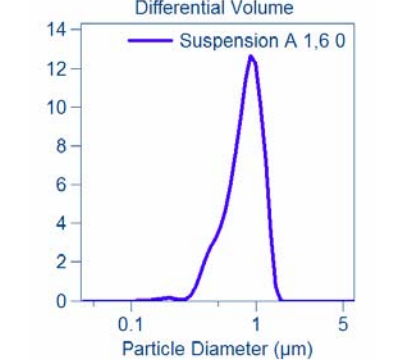
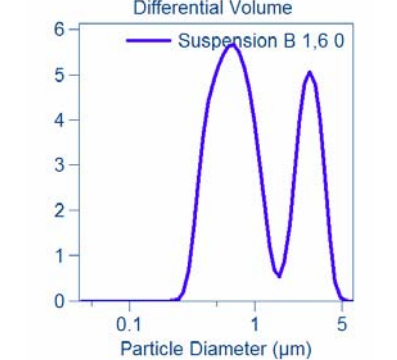
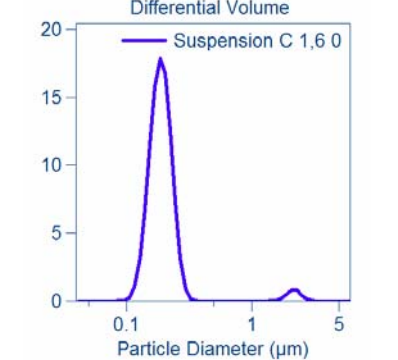
4.5.1 Resolution capacity of the LS 230

Table 4-4 gives an overview of all results obtained from this study. In Fraunhofer analysis mix A was analysed as a monomodal distribution instead of a tetra modal. The obtained peak corresponds to the arithmetic average of 400 and 850nm. The fractions of 100 and 3000nm were not found. Mixture B was analysed as a bimodal distribution instead of a trimodale one. Also here one peak (625nm) corresponds to the average of the two fractions of 400 and 850nm. The fraction of 3000nm was identified correctly. Suspension C is only bimodal, but was analysed to be trimodale. The middle peak at 200nm is correct. The second peak is found at 571nm, but not at 850nm. An additional peak occurs at 70nm. In summary, using Fraunhofer mode did not lead to correct results in any case.

In Mie mode mixture A was detected to be a bimodal distribution instead of a tetramodal distribution. The mean peak is found at 910 nm, which is almost in agreement to the main particle fraction of 850nm added to the mixture. The second peak found is 200nm, which is very little and not correct, as 100nm particles were added to the mixture. In contrast to the Fraunhofer modus, the main peak is broader and a very weak shoulder can be seen at 400nm. The expected peak at 3000nm is totally missing. The comparison of Fraunhofer and Mie mode indicates a more sensitive result, when the Mie mode was used. However the result is very disappointing as no tetra modal distribution could be detected. The smallest fraction of the mixture was detected as to large, whereas the largest fraction is missing at all. The medium sized particle fractions could not be separated.

The analysis of suspension B in Mie mode did not lead to a satisfying result. The two fractions of 400 and 800nm are analysed as an averaged peak at 630nm. The peak at 3000nm was analysed correctly in size. Also suspension C, which was only bimodal could not be analysed correctly. Whereas the main fraction of 200nm was analysed correctly, the second particle fraction was found to be at 2300nm, but not at 850nm, which would correspond to the real mixture. Also the calculated volume distribution is promised by the manufacturer to correspond to the real volume distribution. Here no correct distribution was found for all results analysed with Fraunhofer approximation.

Table 4-4: Overview of results of the nailing test: the known volume distributions of mix A, B and C are shown in Microsoft Excel diagrams, the LD measurements were analysed using Fraunhofer approximation and Mie-mode with the optical parameters for latex (1.6 real part, 0 imaginary part) The LD results are shown in curves with logarithmic ordinate as obtained from the software of the LS 230

Latex Suspension A	Latex Suspension B	Latex Suspension C
expected results		
tetramodal	trimodal	bimodal
100, 400, 800 and 3000nm.	400, 850 and 3000nm	200nm and 850nm
 <p>A bar chart showing the expected volume distribution for Suspension A. The x-axis is 'particle size in nm' with values 102, 404, 845, and 2875. The y-axis is 'volume %' from 0 to 50. The bars represent approximately 18% at 102 nm, 18% at 404 nm, 45% at 845 nm, and 18% at 2875 nm.</p>	 <p>A bar chart showing the expected volume distribution for Suspension B. The x-axis is 'particle size in nm' with values 404, 845, and 2875. The y-axis is 'volume %' from 0 to 45. The bars represent approximately 40% at 404 nm, 20% at 845 nm, and 40% at 2875 nm.</p>	 <p>A bar chart showing the expected volume distribution for Suspension C. The x-axis is 'particle size in nm' with values 204, 845, and 2875. The y-axis is 'volume %' from 0 to 100. The bars represent approximately 95% at 204 nm and 5% at 845 nm.</p>
results of Fraunhofer analysis		
monomodal	bimodal	trimodale
peak at 630nm (average of 400 and 850nm)	peaks at 625 (average of 400 and 850nm) and 3000nm	peaks at 70nm, 200nm, 571nm
 <p>A Fraunhofer analysis plot for Suspension A. The x-axis is 'Particle Diameter (µm)' on a logarithmic scale from 0.1 to 5. The y-axis is 'Differential Volume' from 0 to 20. A single peak is shown at approximately 0.45 µm (630 nm), labeled 'Suspension A Fraunhofer.Sls'.</p>	 <p>A Fraunhofer analysis plot for Suspension B. The x-axis is 'Particle Diameter (µm)' on a logarithmic scale from 0.1 to 5. The y-axis is 'Differential Volume' from 0 to 14. Two peaks are shown: a large one at approximately 0.45 µm (625 nm) and a smaller one at approximately 3.0 µm (3000 nm), labeled 'Suspension B Fraunhofer.Sls'.</p>	 <p>A Fraunhofer analysis plot for Suspension C. The x-axis is 'Particle Diameter (µm)' on a logarithmic scale from 0.1 to 5. The y-axis is 'Differential Volume' from 0 to 12. Three peaks are shown at approximately 0.07 µm (70 nm), 0.2 µm (200 nm), and 0.57 µm (571 nm), labeled 'Suspension C Fraunhofer.Sls'.</p>
results of Mie analysis		
bimodal	bimodal	bimodal
peaks at 200 and 910 nm and weak shoulder at 400nm	peaks at 630 and 2700nm	peaks at 200, 2300nm
 <p>A Mie analysis plot for Suspension A. The x-axis is 'Particle Diameter (µm)' on a logarithmic scale from 0.1 to 5. The y-axis is 'Differential Volume' from 0 to 14. The plot shows a bimodal distribution with a main peak at approximately 0.9 µm (910 nm) and a smaller peak at approximately 0.2 µm (200 nm), labeled 'Suspension A 1,6 0'.</p>	 <p>A Mie analysis plot for Suspension B. The x-axis is 'Particle Diameter (µm)' on a logarithmic scale from 0.1 to 5. The y-axis is 'Differential Volume' from 0 to 6. The plot shows a bimodal distribution with a main peak at approximately 0.63 µm (630 nm) and a smaller peak at approximately 2.7 µm (2700 nm), labeled 'Suspension B 1,6 0'.</p>	 <p>A Mie analysis plot for Suspension C. The x-axis is 'Particle Diameter (µm)' on a logarithmic scale from 0.1 to 5. The y-axis is 'Differential Volume' from 0 to 20. The plot shows a bimodal distribution with a main peak at approximately 0.2 µm (200 nm) and a very small peak at approximately 2.3 µm (2300 nm), labeled 'Suspension C 1,6 0'.</p>

4.5.2 Conclusion

The nailing test for the LS 230 was not successful for the multi-modal distributions analysed. In contrast to the manufacturer, where a complex trimodale latex mixture can already be analysed in the Fraunhofer mode, here none of the tested suspensions was analysed correctly. This indicates that only optimised mixtures will yield highly resolved results. Moreover, it was found that especially larger particles might not be detected in the presence of a smaller bulk population. This is of high interest as the detection of larger particles is crucial for the characterisation of many pharmaceutical suspensions, in this case nanosuspensions and lipid nanoparticles, not only for stability testing, but also for safety reasons. In case systems are intended for intravenous injection, larger particles may cause capillary blockage, which can lead to the death of the tested animals and humans.

Therefore these findings need to be investigated in more detail, especially for systems e.g. nanosuspensions and lipid nanoparticles.

4.6 Influences of optical parameters on results

4.6.1 Analysis of polydisperse systems

Even though the manufacturer describes the influence of the analysis mode used as little (Beckman-Coulter 1994), it was seen from the nailing test that the change of the analysis mode led to remarkable changes of the result analysed. In order to understand to which extend the results are influenced by a change of the optical parameters, the influence of different parameters, relevant for the analysis, was investigated. This was possible as the software of the LS 230 allows changes in the analysis mode and the optical parameters any time after the measurement itself was performed. The raw data are then calculated again, based on the new parameter settings. This enables a direct comparison of influences due to changes of optical parameters by using only one measurement. Values used for the simulations for the real part of the refractive index ranged from 1.35 – 1.8, values for the imaginary part ranged from 0-3 (see Table 4-5)

The simulation was performed by combining every imaginary part with every real part.

Table 4-5: Overview values of optical parameters used for simulation

values used for real refractive index	values used for imaginary refractive index
1.35	0
1.40	0.001
1.45	0.01
1.50	0.03
1.55	0.05
1.60	0.10
1.65	0.30
1.70	0.50
1.75	1.0
1.80	3.0

4.6.1.1 Influence of parameter setting on diffraction pattern

In order to understand the mechanism of a change in optical parameters, the influence of the obtained diffraction pattern was investigated. The diffraction pattern corresponds to the detected intensity. It was checked if changes in detected intensities in the instrument occurred (e.g. a decrease of the total intensity detected, when the imaginary value is changed) when the optical parameters were changed. There was no influence on the intensity detected, when optical parameters were changed.

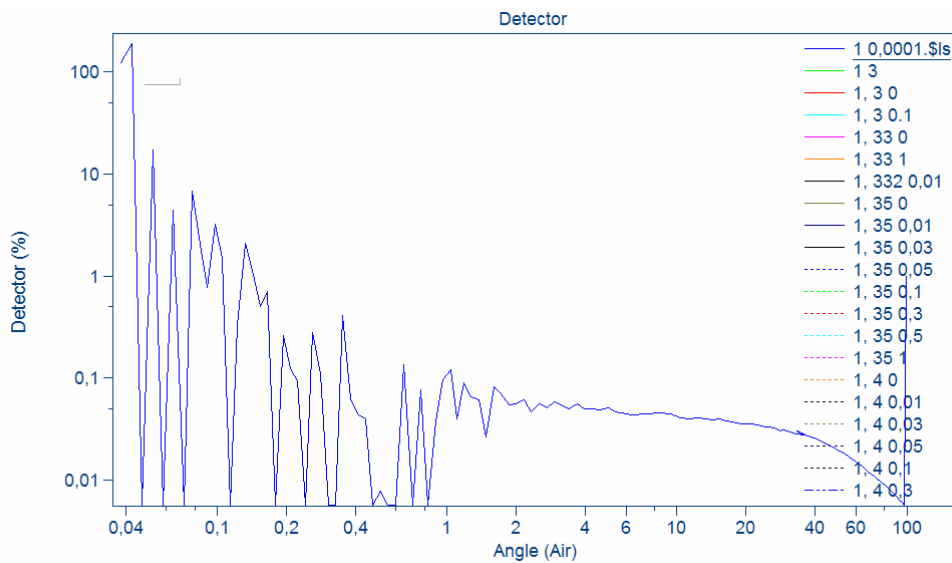


Figure 4-8: Overlay of intensities detected of all simulations, changes in optical parameters have no influence on the intensity detected

4.6.1.2 Influence on PIDS data

Also the PIDS data are not influenced by the change of optical parameters.

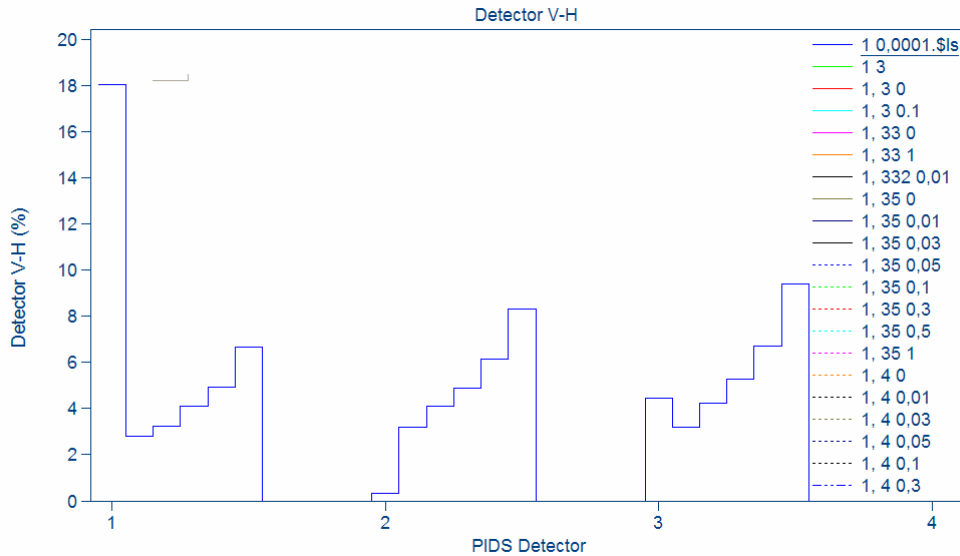


Figure 4-9: Overlay of PIDS data of all simulations, changes in optical parameters have no influence on the data

4.6.1.2.1 Conclusion

Changes in optical parameters do not change the diffraction pattern and have no influence on the PIDS data which are obtained. The results clearly show that the intensities correspond to the raw data, hence the truly detected intensities in the instrument. Detected data cannot be influenced by changes in the software. What actually happens to the data if parameters are changed cannot be understood from this set of data. The algorithm used for the analysis of the raw data is a manufactures secret.

4.6.1.3 Influence of optical parameters on particle size and particle size distribution

Neither the flux intensities of diffraction nor the PIDS intensities of the measurements are changed, when optical parameters and/or the analysis mode were changed. From the nailing test it was found that there is a strong influence on the particle size and the particle size distribution when parameters are changed. In order to investigate the influence of those changes in more detail, the measurements from the nailing test were simulated by using a large variety of optical parameters (see Table 4-5).

The results are arranged this way, that influences become visible when the imaginary part (imaginary refractive index - IRI) is increased and the real part (real refractive index - RI) is kept constant (Figure 4-10, Figure 4-12, Figure 4-14). In order to investigate the influence of the imaginary part, also graphs were established where the real part (BI) is increased and the imaginary part (IBI) is kept constant (Figure 4-11, Figure 4-13 and Figure 4-15).

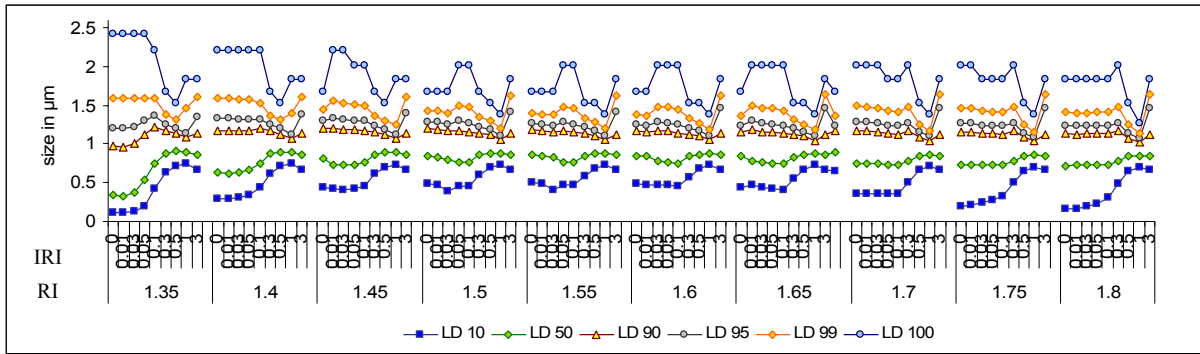


Figure 4-10: Simulation Latex mix A (RI) – real refractive index (RI) was kept constant in each respective set, sets calculated for RI from 1.35-1.8 (left to right) and imaginary part (IRI) was varied from 0 to 3

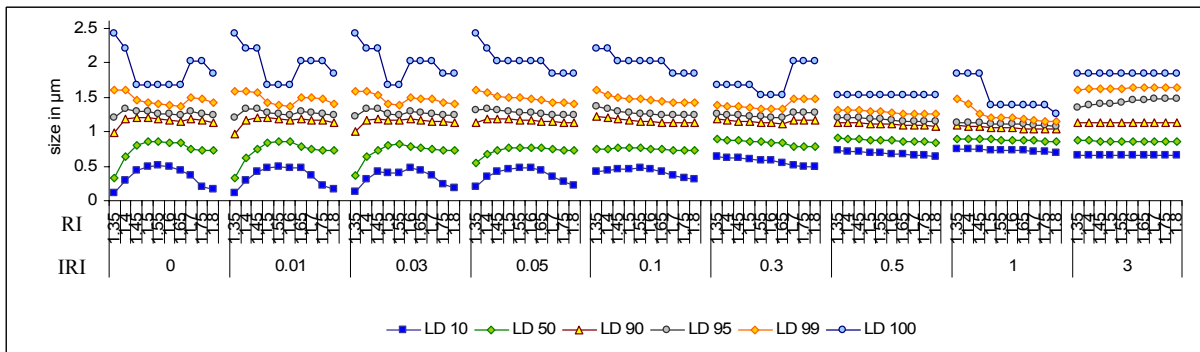


Figure 4-11: Simulation Latex mix A (IRI) - IRI was kept constant in each respective set, sets calculated for IRI from 0-1, RI was varied from 1.35-1.8

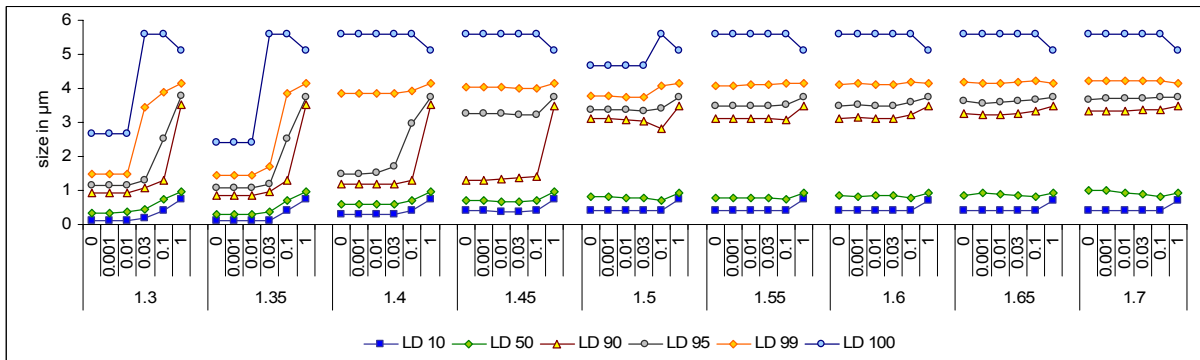


Figure 4-12: Simulation Latex mix B (RI) – as in Figure 4-10

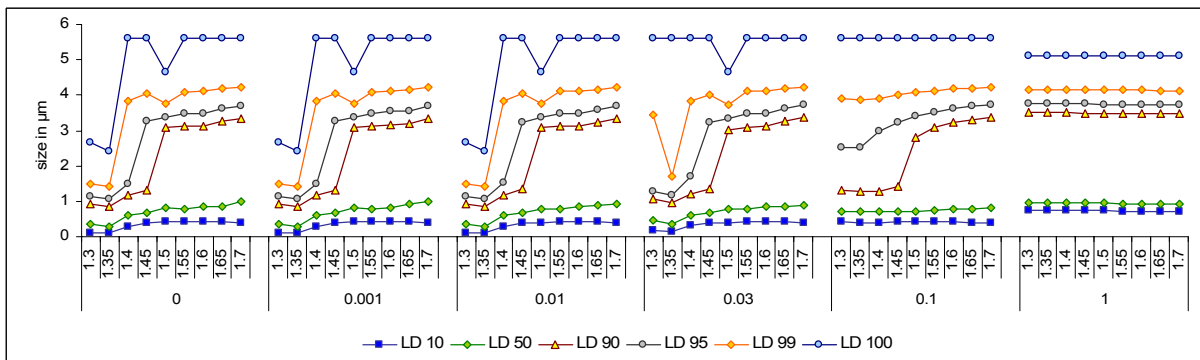


Figure 4-13: Simulation Latex mix B (IRI) - as in Figure 4-11

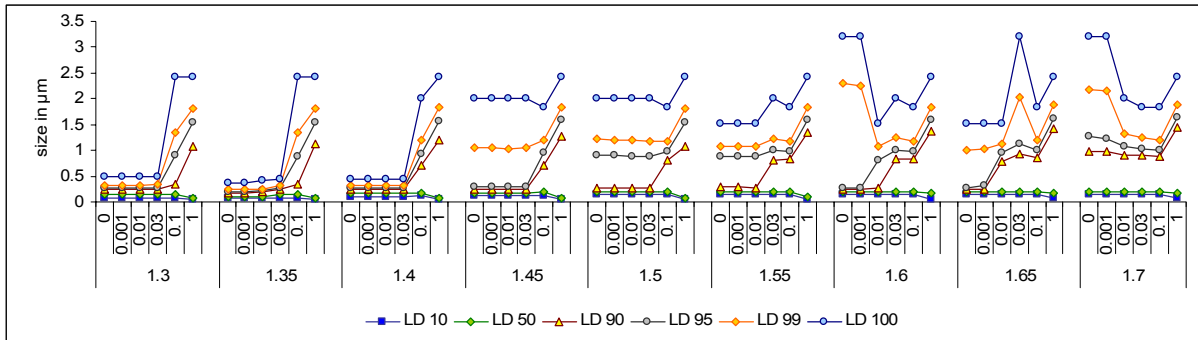


Figure 4-14: Simulation Latex mix C (RI) – as in Figure 4-10

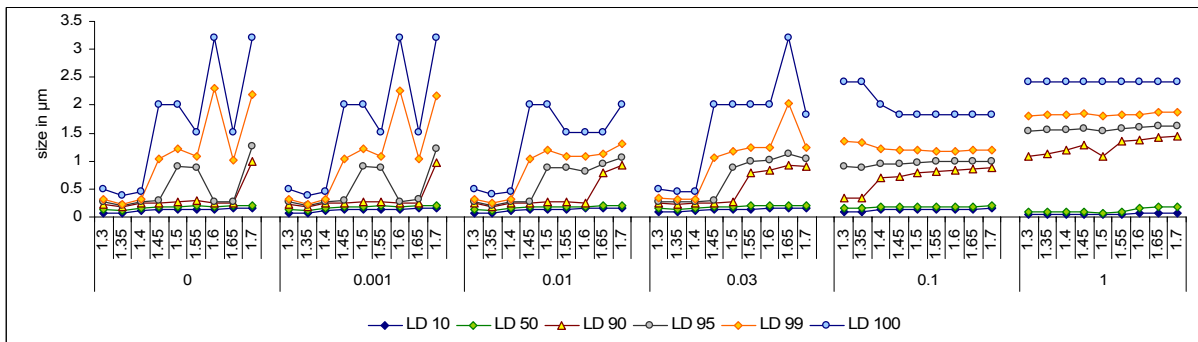


Figure 4-15: Simulation of latex mix C (IRI) - as in Figure 4-11

The influences of changes in RI and IRI on the LD values are not similar in the three mixes analysed. However, in general the differences for all LD values between the different optical parameters are unexpectedly high. In all mixtures the LD 100 was the most affected value.

In latex mix C the LD 10 and LD50 values were influenced only to a small amount, whereas the values of LD 90 - LD 100 increased with an increase in RI. Latex mix C was a bimodal distribution, with a bulk population of 200nm and a smaller side population of 850nm. In the analysis the detection of the bulk population was always given. However the detection of the side population varied. In some of the simulation results no side population was detected, which leads to small values in LD 90 – LD 100, but values of LD 10 and LD 50 are not much influenced. In some other cases the side population was detected as being too large (i.e. 2700nm). Also here the LD 10 and LD 50 are not much influenced, but LD 90 - LD100 were increased dramatically.

In mix A, which was a tetramodal distribution influences are less dramatic, when compared to the latex mixtures B and C. Here the analysis only led to a monomodal distribution in each optical model used, where only the broadness and the position of the main peak changed to a small amount.

In latex mix B the change of optical parameters led to the greatest changes. It was a trimodal distribution, consisting of 40w% of small particles (400nm), 20w% of medium sized particles

(850nm) and 40w% of larger particles (2775nm). Most results from the simulation only detected a bimodal distribution were either no larger particles or no smaller particles were found. Therefore the influence of the optical parameters on the size is much more pronounced than it was found for the simulations of mix A and C.

Interestingly for suspension B no correct result was obtained using the correct parameters for latex particles (i.e. RI = 1.6, IRI = 0). But using the parameters 1.5 and 0 led to the correct resolution for the detection of the three different particle populations, however the volume distribution was incorrect (Figure 4-16). Figure 4-16 also highlights the influence on the optical parameters on the particle distribution. Depending on the optical parameter the size distribution may vary from monomodal, bimodal or even trimodale.

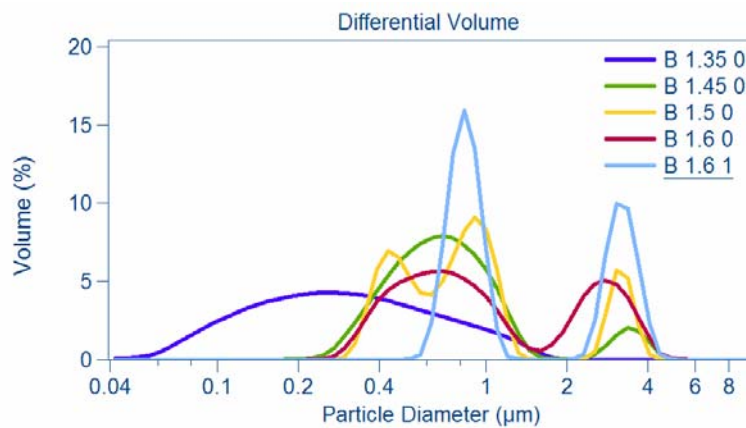


Figure 4-16: Overlay of particle size distributions of latex mix B obtained from different optical parameters. The best resolution was found for the optical values of 1.5 (RI) and 0 (IRI) which do not correspond to the known optical parameters of latex particles being 1.6 for RI and 0 for IRI.

In order to demonstrate the outstanding impact of the optical parameters on the results obtained in a condensed and simple overview, the gained minimal and maximal values for LD 10 - LD 100 are listed in Table 4-6. It shows that for instance the LD 50, often referred as the mean particle size of a sample, varied between 330nm and 905nm (Mix A). In Figure 4-17 the absolute differences of minimal and maximal values are shown. Clearly demonstrating that the information of many manufactures, saying optical parameters have no big impact on the total result, is false.

Table 4-6: List of minimal and maximal values due to changes in optical parameters, obtained for LD 10, LD 50, LD 90, LD 95, LD 99 and LD 100 in μm

		minimal size in μm	maximal size in μm
MIX A	LD 10	0.116	0.745
	LD 50	0.330	0.905
	LD 90	0.961	1.223
	LD 95	1.078	1.468
	LD 99	1.142	1.636
	LD 100	1.261	2.423
MIX B	LD 10	0.106	0.748
	LD 50	0.284	0.996
	LD 90	0.842	3.510
	LD 95	1.067	3.763
	LD 99	1.435	4.226
	LD 100	2.423	5.610
MIX C	LD 10	0.051	0.153
	LD 50	0.079	0.199
	LD 90	0.173	1.439
	LD 95	0.195	1.628
	LD 99	0.235	2.305
	LD 100	0.375	3.206

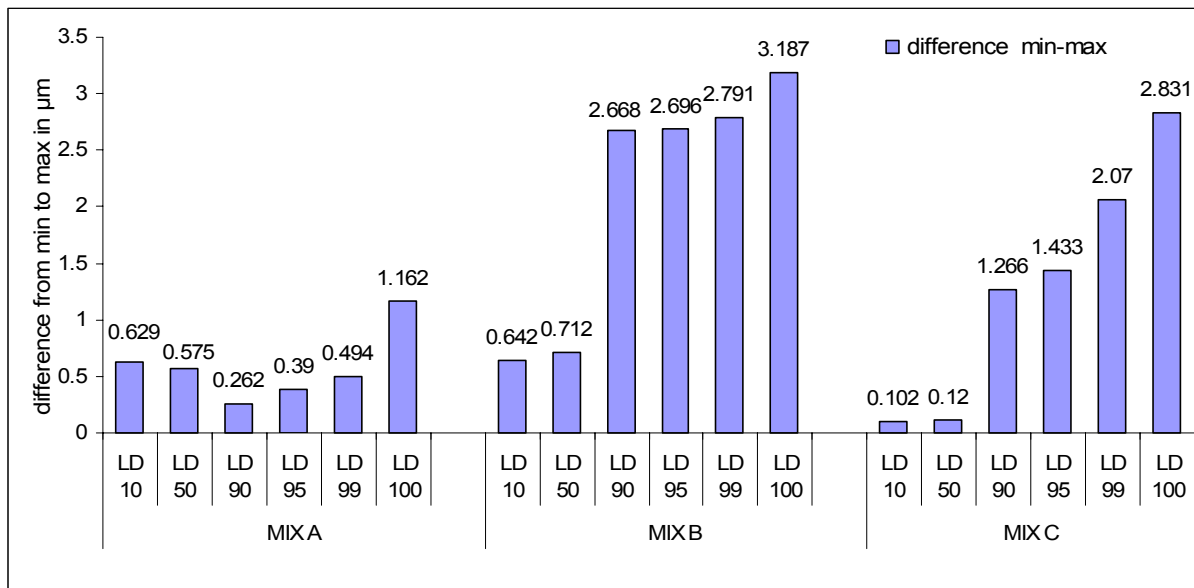


Figure 4-17: Overview of differences between minimal and maximal values for the different LD in μm

4.6.1.3.1 Conclusion

The results show the importance of the refractive index in respect to the result obtained. Results vary tremendously, indicating that the results are strongly influenced by the choice of the optical parameters used. The results of the different simulations do not show any regular

or similar changes when varying the optical parameters. Only one trend was observed. It was found, that the influence of the real refractive index decreases, when the imaginary part is increased. At very high imaginary values (i.e. >1) the real part has no impact on the result analysed anymore (Figure 4-11, Figure 4-13 and Figure 4-15). However, such high IRI values do not correspond to real sample properties. Typical correct IRI values for nanosuspensions and NLC are in the range from 0.001 to 0.3 that means to obtain a correct result precise knowledge of the RI of the systems is essential!

4.6.1.4 Influence on the width of the distribution

The width of the distribution was estimated by calculating the nominal standard deviation

$$\left(SDn = \frac{\sum \sqrt{(x - \bar{x})}}{n} \right).$$

Also here the changes are tremendous and irregular. The trend found from the particle size is also found here. The influence of the real part decreases with an increase of the imaginary part.

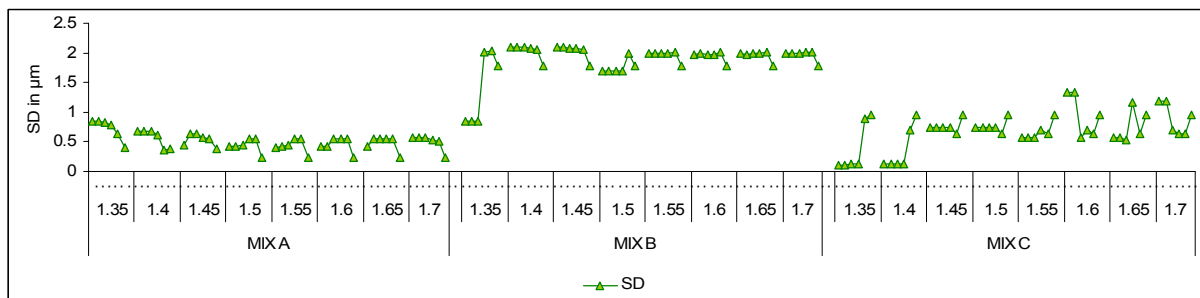


Figure 4-18: Influence on the width of particle size distribution by variation of the optical parameters – real refractive index (RI) was kept constant in each respective set, sets calculated for RI from 1.35-1.7 (left to right) and imaginary part was varied from 0 to 3 (values shown as ...)

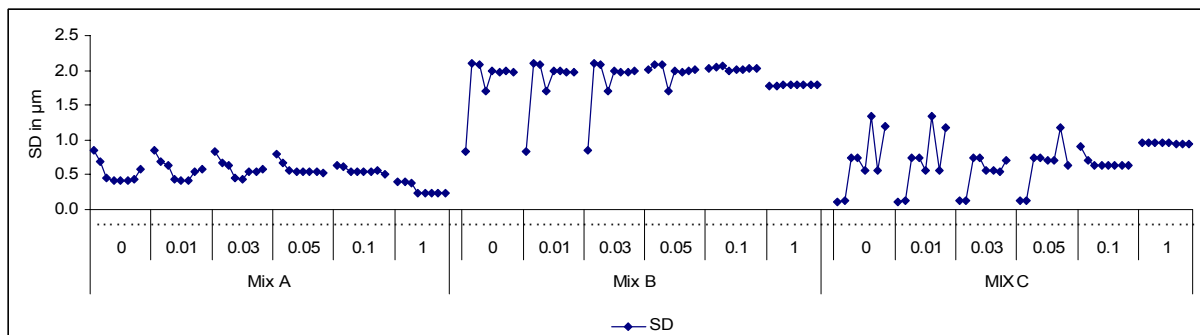


Figure 4-19: Influence on the width of particle size distribution by variation of the optical parameters – imaginary refractive index (IRI) was kept constant in each respective set, sets calculated for IRI from 0-1 (left to right) and real refractive index was varied from 1.35 to 1.7 (values shown as ...)

4.6.1.5 Influence on results using different size distributions

The analysed size distribution can be viewed as a volumetric distribution, as well as surface specific or number distribution. In general analysis from LD measurements are given as a volume distribution, however in some cases the numeric distribution can be used. In the nailing test the suspensions have been analysed only as a volumetric distribution. Here the same measurements are also viewed as surface specific and numeric distribution. Figure 4-20 gives an overview of some selected data with different optical parameters. In principle, also here only the correct index of refraction yields results with closest proximity to the real mix. Fraunhofer approximation led to the least correct results. An exception is suspension B, where the best resolution was yielded when 1.5 was used for the real refractive index and 0 for the imaginary part (second row, yellow line).

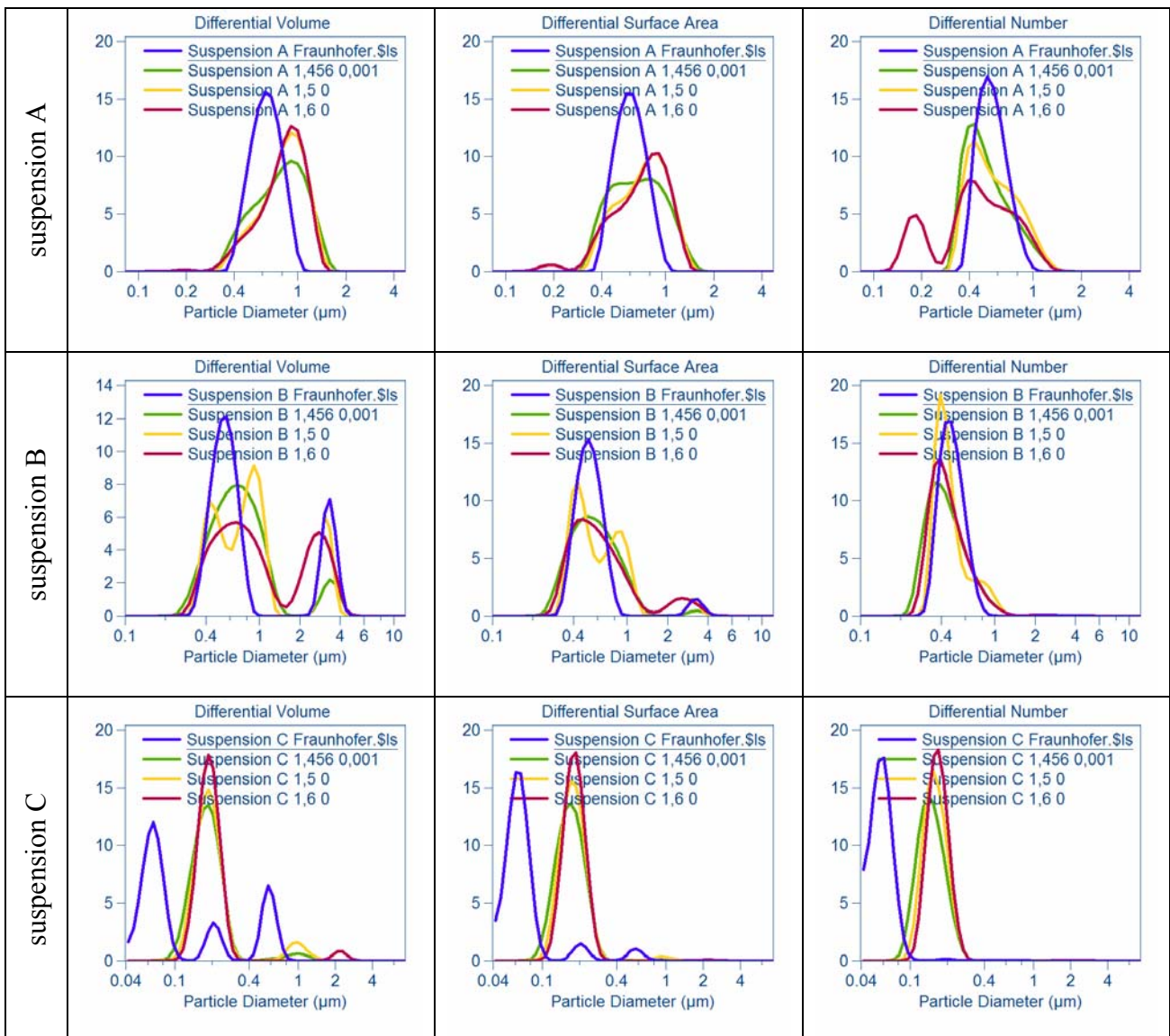


Figure 4-20: Particle size distribution of suspension A (upper row), B (middle row) and C (lower row) plotted as volumetric distribution (first column), surface specific and numeric distribution (second and third row), numeric distribution increases the impacts of smaller particles, larger particles loose impact

The comparison of the different distribution modes used clearly shows the strong influence of this parameter on the particle size obtained. As seen from the different graphs, the analysis in surface specific distribution shifts the size graph towards smaller particle sizes, whereas the impact of smaller particles increases. The impact of smaller particles is even more increased when the results are viewed as numeric distribution. In suspension A (upper row) the small peak at 200nm is almost not visible in case of the volumetric distribution, in the numeric distribution clearly three fractions of particles are detected (RI 1.6, IRI 0). This result indicates, that if small particles exist within a sample besides larger particles, they can easier be detected by analysing the sample with a numeric distribution if the volumetric distribution does not lead to its detection.

The comparison of the results obtained from different distribution modes for suspension B and C indicates the problem of this kind of analysis. It shows, that larger particles escape from detection when the results are analysed in a surface specific or numeric distribution, e.g. in suspension B, where particles of 3000 μm are present within the volumetric distribution, but not any more in the numeric distribution (second row), right. In conclusion the analysis as numeric distribution can lead to the detection of small particles besides a larger main population, but the existence of larger particles besides a small sized bulk population might be overseen. Therefore it is suggested, that numeric distribution analysis should only be applied if small particles are expected, but could not be detected with the standard volumetric distribution mode. Numeric analysis alone is not sufficient; if it is used also the results from the volumetric distribution should be included into the report, as the use of numeric distribution was found to be not sensitive enough for the detection of larger particles.

4.6.1.6 Summary

From all the results it is concluded, that changes in the optical parameters affect the particle size diameters, the width of the distribution as well as the shape of the distributions in a pronounced way. A trimodal distribution may change into a bimodal or even monomodal distribution, depending on the optical module used. All this leads to incorrect results. Therefore a correct particle size characterisation of submicron particles by using laser diffractometry can only be done when the indices of refraction are known.

4.6.2 Analysis of monodisperse latex dispersion

As already mentioned the accurate index of refraction is very hard to obtain for solids and drug powders. Some people even believe it is not possible at all (Beckman-Coulter 1994). Apart from this fact, particle sizing by laser diffractometry is frequently performed and

submicron particles or microparticles below the Fraunhofer range are not excluded from this method. Therefore it needs to be assumed that in practice optical parameters are often guessed. Many people rely on the value found from R. Schumann which is 1.456 for the real part and 0.001 for the imaginary part (for details it is referred to (Müller 1996). It is often overseen that the value was found for intravenous fat emulsions, where the particles consist of soy bean oil and phospholipids. If the correct index of refraction is not known and guessed values are used for the real and imaginary refractive index, the following 9 combinations can be obtained: see Table 4-7

Table 4-7: The nine possible combinations of optical parameters if the correct ones are not known: in bullet points (left) table with the numbers derived from the numbers of the bullet points (right)

<ol style="list-style-type: none"> 1. both indices are too small 2. real part is too small, imaginary part matches 3. real part is too small, imaginary part is too big 4. real part matches, imaginary part is too small 5. both indices are matching 6. real part matches, imaginary part is too big 7. real part is too big, imaginary part is too small 8. real part is too big, imaginary part matches 9. both indices are too big 	IRI	too small	ok	too big
	BI			
	too small	1	2	3
	ok	4	5	6
	too big	7	8	9

A monomodal latex dispersion was analysed and simulated (Latex Paulke, batch code: BMG 22, 1.7µm (PCS, z-average)). Optical parameters from the literature are 1.6 or 1.59 for the real part and 0 or 0.1 for the imaginary part of the refractive indices (Beckman-Coulter 1994; Chen, Kromin et al. 2003). In order to investigate the trends in changes on the result caused by too high or too low optical parameters, the values from the literature were increased or decreased. The measurement was simulated by using larger and smaller values for the known optical parameters. The values chosen for the experiment here derived from the knowledge, that organics typically have real refractive indices ranging from 1.3 -1.8 because of its carbon skeleton (Malvern 2004). Imaginary values range from 0-0.1 if the material is transparent. It increases to 1 if the compound is white and opaque. For grey or coloured compounds the imaginary part can further increase and reaches a maximum of 10 for compounds such as soot (Beckman-Coulter 1994; Malvern 2004). Selected values for the imaginary refractive index ranged from 0-1, because only transparent material was used. 1.4 was chosen for a small refractive index, 1.75 and 1.8 were chosen for large refractive indices (Table 4-9). The system

itself was kept easy in its composition, in order to obtain clear trends of effects. Measurements were carried out under standard conditions after the handbook of the LS 230 (3 repeated runs, measurement time 60s, PIDS included, PIDS obscuration 45%). Additionally the measurement was simulated applying the different RI and IRI combinations.

4.6.2.1 Simulation of mono disperse latex dispersion

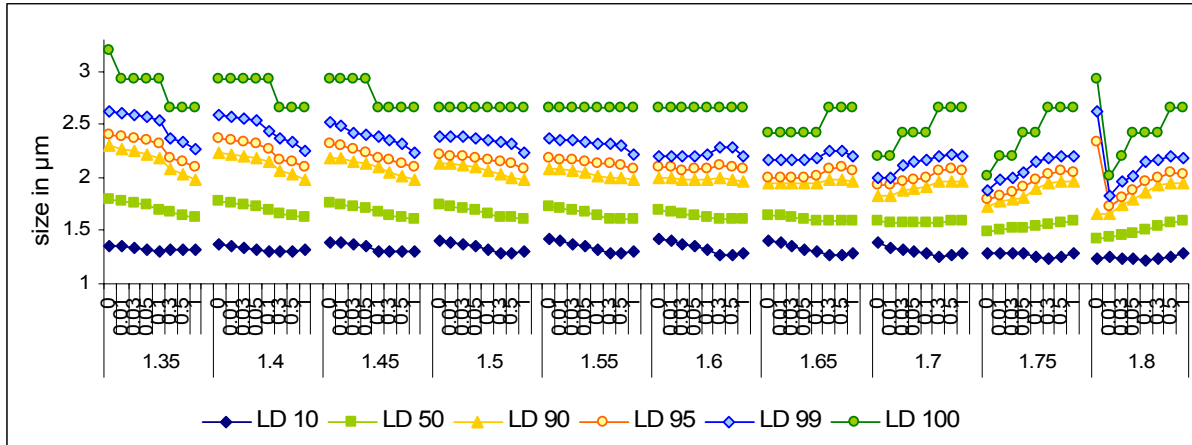


Figure 4-21: Particle sizes obtained from the latex dispersion by changes in optical parameters – real refractive index (RI) was kept constant in each respective set, sets calculated for RI range from 1.35-1.8 (left to right) and imaginary part (IRI) was varied from 0 to 1

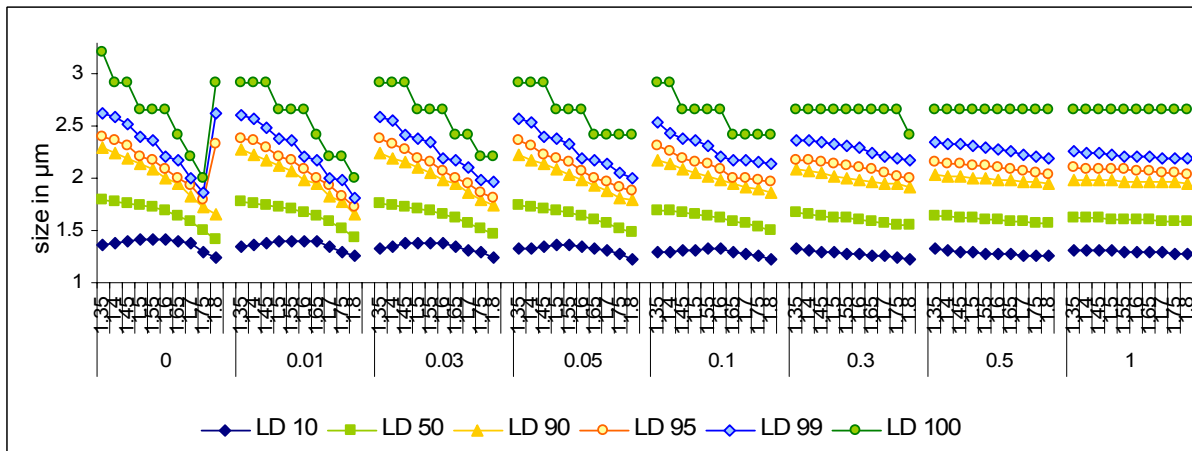


Figure 4-22: Particle sizes obtained from the latex dispersion by changes in optical parameters– imaginary refractive index (IRI) was kept constant in each respective set, sets calculated for IRI from 0-1 (left to right) and real part (RI) was varied from 1.35 to 1.8

The simulations from the measurement also show a strong influence on optical parameters (Figure 4-21 and Figure 4-22). Minimal and maximal values are listed in Table 4-8. Maximal differences in LD 50 are 0.348 μm and even 1.195 μm for the LD 100.

Table 4-8: Minimal and maximal values (second and third row) and the difference of those (fourth row) for the simulation of monodisperse latex dispersion BMG 22 (PCS z-average 1.7 μ m)

max	LD 10	LD 50	LD 90	LD 95	LD 99	LD 100
min	1.226	1.439	1.656	1.734	1.821	2.011
max	1.417	1.787	2.297	2.409	2.63	3.206
difference	0.191	0.348	0.641	0.675	0.809	1.195

The simulation in Figure 4-21 shows a trend. When the RI is small the particle size decreases when IRI is increased. At medium values for RI the size is relatively consistent, whereas at larger values for RI the size increases when the imaginary part (IRI) is increased. The simulation in Figure 4-22 again shows that the influence of the refractive index decreases with an increase in the imaginary part. At high values the influence becomes neglect able.

4.6.2.2 Simulation principle

In order to investigate the principle of changes in particle size caused by the variation of optical parameters, the nine possible combinations (viewed in Table 4-7) were applied to the latex dispersion analysed. The indices used as too small, correct and too large are listed in Table 4-9.

Table 4-9: Optical values used for the simulation of the latex dispersion BMG 22

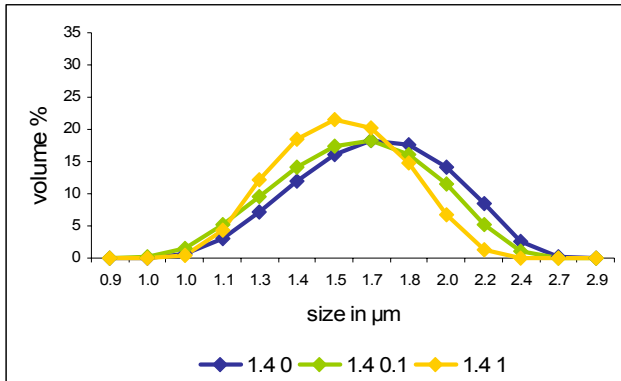
	Real refractive index	Imaginary refractive index
too small	1.4	0
correct	1.6	0.1
too big	1.8	1

In Table 4-10 all size graphs obtained from the simulation are listed. The left column shows the results, when the real refractive index was kept constant and the right column shows the changes in size and size distribution, when the imaginary part is kept constant. The data can be interpreted as follows: when the real part is too small the graph shifts to the left side, when the imaginary part is increased (picture 1). The same effect was seen when the value for the real part was correct, however the extent of shift was significantly smaller (picture 2). In picture 3 the real part is too big, here the shift observed is from right to left, when the imaginary part is increased.

Picture 4 shows the changes, when the imaginary part is small and the real part is increased. Here the curve shifts from right to left, which means that particles become smaller when RI is increased. Moreover, also the analysed volume of the population was found to be increased when RI is increased. With higher imaginary parts (picture 5) the effect observed is the same as in picture 4, but the impact is much smaller. When the imaginary part is high (picture 6) almost no differences can be seen in the graphs, indicating also here, the influence of the real part becomes almost zero, when the imaginary part is high.

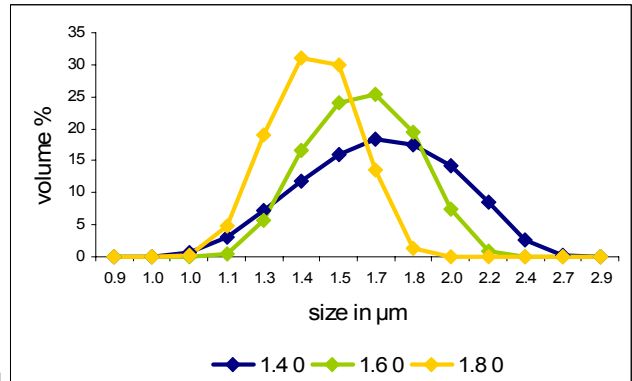
Table 4-10: Size distributions overlay of graphs obtained from the simulation of a monomodal latex dispersion (batch code: BMG 22) with different optical modals left column: RI constant (1.4 – upper, 1.6 – middle, 1.8 – lower) and the IRI increases (0, 0.1, 1) right column: IRI is constant (0 – upper, 0.1 middle, 1 – lower) and RI increases (1.4, 1.6, 1.8)

RI is kept constant and IRI increases

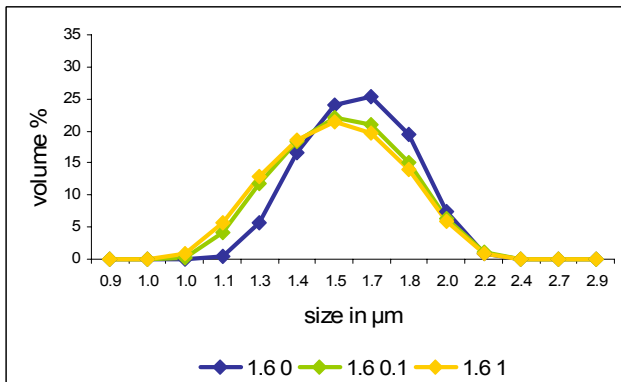


1 Influence on particle size if real part is too small and imaginary part is changed; size decreases, with an increase of the imaginary part

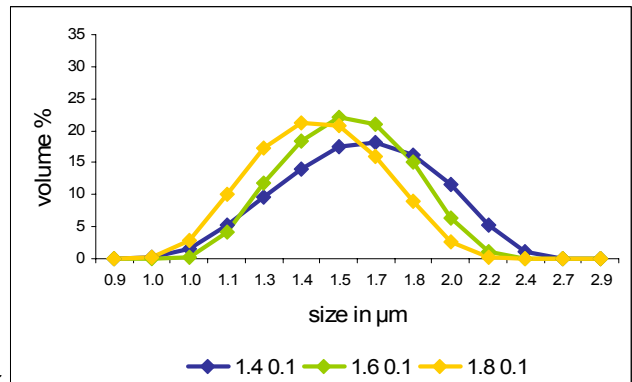
IRI is kept constant and RI increases



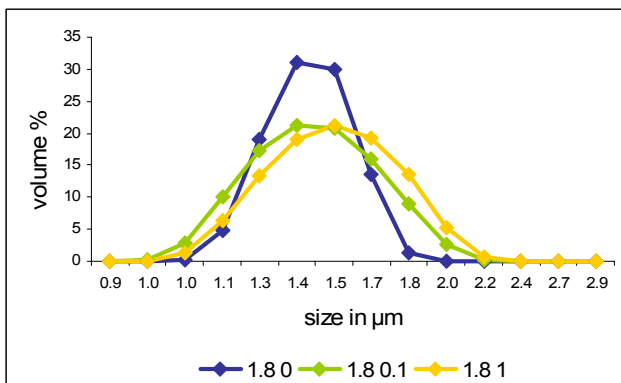
4 Influence on particle size if real part is too small and imaginary part is changed; size decreases, with an increase of the imaginary part



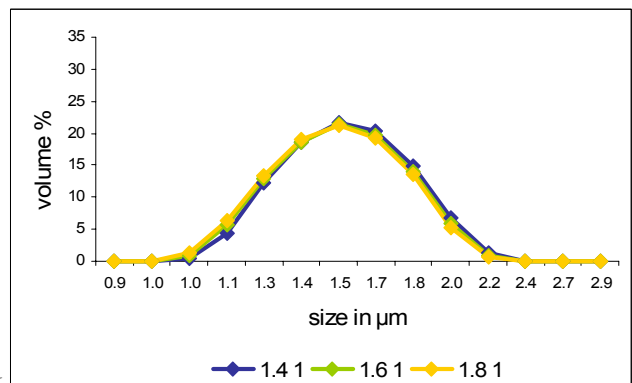
2 Influence on particle size if real part matches and imaginary part is changed; size decreases, with an increase of the imaginary part, changes are much smaller than seen in 1.



5 Influence on particle size if real part matches and imaginary part is changed; size decreases, with an increase of the imaginary part, changes are much smaller than seen in 4.



3 Influence on particle size if real part is too big and imaginary part is changed; size increases, with an increase of the imaginary part, opposite as seen in 2.



6 Influence on particle size if real part is too big and imaginary part is changed, almost no differences in sizes can be obtained.

Table 4-11 summarises the observations found. The summary is only valid for monomodal size distributions. If polydisperse systems are analysed, the trend is not clearly visible.

Table 4-11: Summary of trend in changes of particle size observed when the mono modal latex dispersion was simulated (cf. also Table 4-10) symbols: \emptyset = mean diameter, \uparrow = increase, $\uparrow\uparrow$ = strong increase, \downarrow = decrease, $\downarrow\downarrow$ = strong decrease, $=$ = no change

	if	than
RI 1.4	IRI \uparrow	\emptyset $\downarrow\downarrow$
RI 1.6	IRI \uparrow	\emptyset = or $\uparrow\downarrow$
RI 1.8	IRI \uparrow	\emptyset $\uparrow\uparrow$
IRI 0	RI \uparrow	\emptyset $\downarrow\downarrow$
IRI 0.1	RI \uparrow	\emptyset \downarrow
IRI 1	RI \uparrow	\emptyset =

From these data and also from the previous simulations (cf. Table 4-4 (p.60), Figure 4-16 (p.66) and Figure 4-20 (p.69)) it could be seen that also the volumes of the detected particle populations are influenced when the optical parameters are changed. The trend here was, that the intensity increases with an increase in the imaginary values, when the real part is small. At high real refractive indices, the intensity decreases if the imaginary part is increased. When the imaginary part is kept constant, the intensities decrease when the real part is increased. The influence becomes smaller, the higher the imaginary value is.

4.6.2.3 Interpretation of data

The refractive index is defined as the ratio of the velocity of light in different optical media.

The higher the refractive index, the more is the incident light reflected towards to the perpendicular (i.e. the angle becomes smaller) (Haas 2002). If particles in the submicron range are analysed by laser diffractometry, not only diffracted light but also refracted light is detected on the detectors of the instrument, as particles have a lower forward scattering intensity.

If Mie theory is used for analysis, and the correct refractive index is used, the intensity of detected light, which comes not from diffraction, but from refracted light, has to be subtracted from the intensity data originally detected. Otherwise the additional intensity from refraction would be incorporated into the Mie diffraction analysis, leading to a different pattern analysed and to different results therefore. Figure 4-1 shows a schematic image of a scattering pattern obtained on the detector during analysis. The point X corresponds to a light intensity from refracted light. If the right index of refraction is used, this intensity is cancelled out from the analysis by the software. The real pattern of diffraction is obtained and can be analysed using the Mie formula.

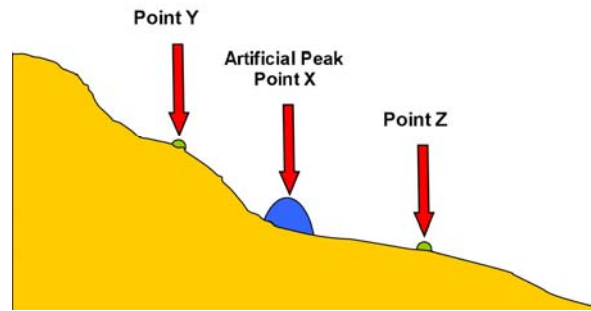
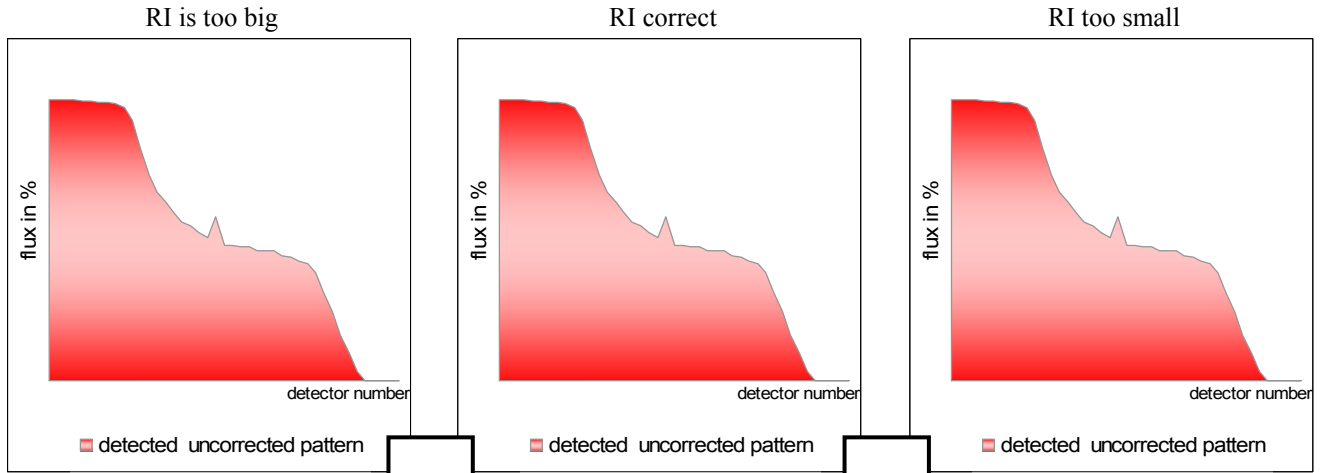


Figure 4-23: Schematic diffraction pattern of a polydisperse sample point X= light from refracted light, point Y= light will be cancelled here, if index of refraction is set to be too big, point Z= light will be cancelled here, if ends of refraction is set to be too small

If one imagines, that the constitution of refraction calculated by the software is not correct (due to input of wrong RI), light will be cancelled out from a different part of the detected pattern. Light from the refracted light is not cancelled at the correct position in the detected pattern (point X) but at a different (= wrong) position (e.g. Y or Z)). Therefore, a different pattern of diffraction is generated by the software, leading to different results in analysis. If the value of the refractive index is too large, the intensity is cancelled more towards the middle of the detector (point Y; as higher the refractive index, as smaller is the angle). If the index of refraction is set too small, the software falsely subtracts light intensity at the larger angles (point Z). As pointed out in the introduction, the smaller the particles, the larger is the angle of diffraction. An obtained pattern of diffraction can be nicely compared with a stone which is vertically dropped into water. It produces lobes around it, similar to the Fraunhofer diffraction rings. The first lobe is highest and the intensity decreases with an increasing distance. This is similar to diffracted light, where the first lobe incorporates 75% of the total energy. However, in case a large stone drops into water; one will see a big splash with very narrow lobes occurring next to the stone. A very small stone, when it drops into water, one sees very little waves. Also the distance from the stone to the fist lobe is much broader. These two characteristics are important for laser diffractometry. As described above, the minimum of the first lobe is at $1,22\lambda/d$. The intensity decreases linearly with a decrease in diameter. Because point Y is more close to the middle of the detector, it is assumed, that especially here the first intensities are influenced. Therefore if the real part is set as too big, the calculated particle size will be smaller than in reality because the software has falsely subtracted light intensity in the detected pattern due to the wrong RI value. If the real part is set too small (point Z), the particle size is analysed as too large, because intensities of smaller particles are cancelled from the scattering pattern (Table 4-12).

Table 4-12: Suggested scheme of corrections for the detected patterns by the software based on input of RI and the influence on the particle size analysed for monomodal distributions

1. Detected patterns (=raw data = diffraction and refraction) for

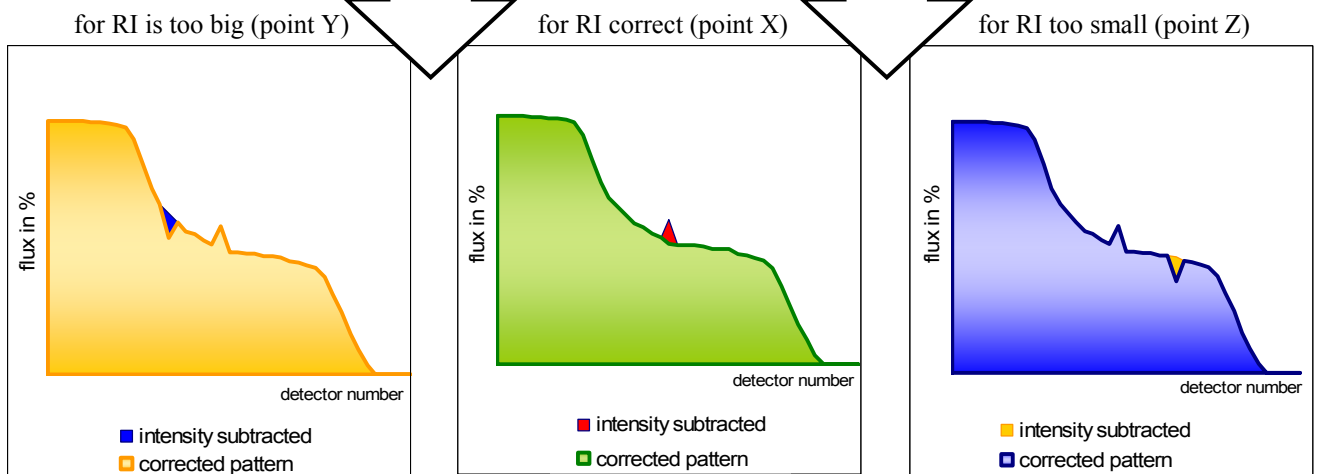


Correction of the detected intensity calculated by the

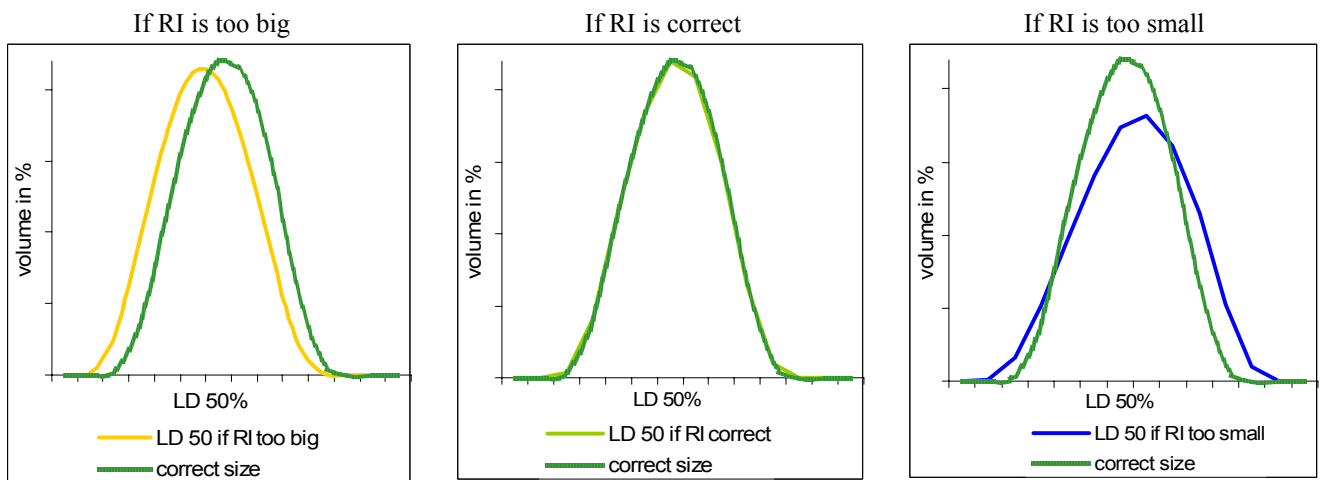
patterns for refracted light software based on input of

by subtraction of refractive RI yields 2.

2. corrected patterns



3. analysed particle size



the particle size is calculated as too small.

the particle size is calculated correctly.

the particle size is calculated as too large.

4.6.2.4 Influence of optical parameters on the particle concentration analysed by the software

The software not only calculates the particle size distribution but also the concentration (v%) of the particles in the measuring medium. As already explained, the sample needs to be diluted for the measurement. In case of the LS 230 the volume of the measuring medium in which the sample will be diluted is 125ml. The amount of sample required depends on the particle size and the concentration of the sample, as well as on the measuring parameters. Measurements with PIDS require sample volumes ranging from 10 μ l-100 μ l, measurements without PIDS require much more (e.g. 500 μ l-5ml). The aim of the experiment was to investigate if and to which extent the concentration analysed by the software is influenced by changes in the optical parameters. The results obtained are shown in Figure 4-24 and Figure 4-25. In Figure 4-24 RI was kept constant in each respective set and IRI was varied. In opposite Figure 4-25, were IRI was kept constant for each set and RI was varied.

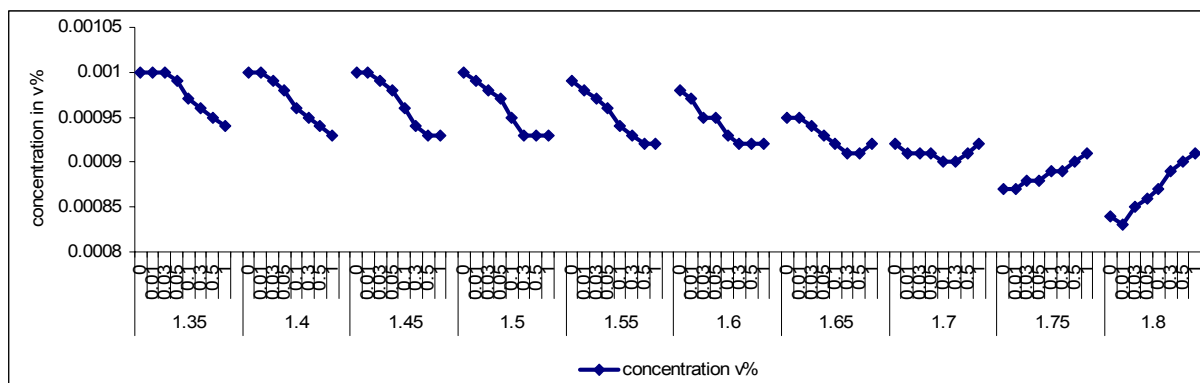


Figure 4-24: Variation of the concentration analysed by the software due to changes in the optical parameters – RI was kept constant for each respective set and IRI was varied from 0-1

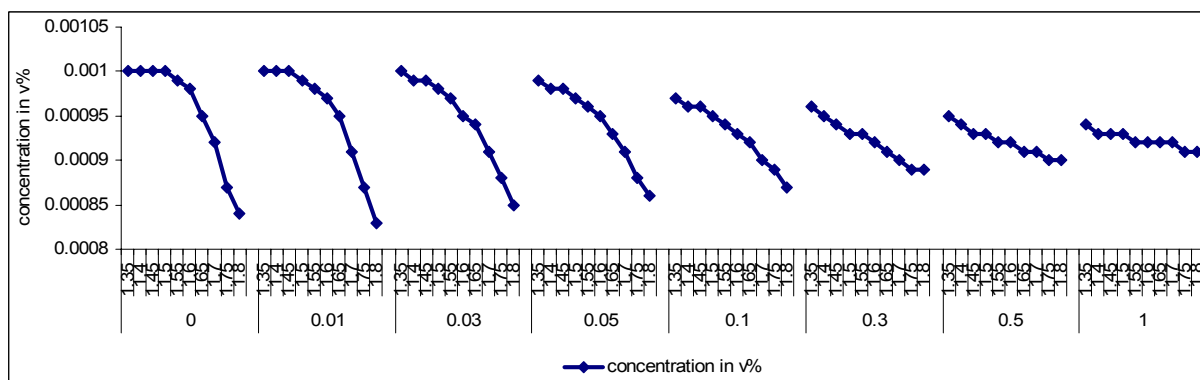


Figure 4-25: Variation of the concentration analysed by the software due to changes in the optical parameters – IRI was kept constant for each respective set and RI was varied from 1.35-1.8

Also the sample concentration calculated by the software is influenced by the optical parameters. From Figure 4-24 it can be seen that the concentration decreases with an increase in the imaginary value, when the real part is small. At higher values for the real part (1.6-1.7) the trend changes, the concentration curve now reaches a minimum at medium imaginary

values. At high values for RI (1.75-1.8) the trend changes again, now the calculated concentration increases with an increase in imaginary part. In Figure 4-25 the change in concentration is shown when the imaginary part is kept constant. Here the concentration decreases, when the real part is increased. The higher the imaginary part, the lower is the influence of the real part on the concentration calculated by the software.

From the data it can be seen that the concentration changes with a change in the optical parameters. As seen from the previous simulations it is clear that also the concentration is only analysed correctly, if the correct optical parameters are used. However in contrast to the particle size and particle size distribution the concentration is not an unknown parameter and can be analysed by various techniques (e.g. UV/Vis spectroscopy or HPLC). If the concentration is known it is possible to vary the optical parameters until the calculated concentration of the software corresponds to the known concentration. The identification of the unknown refractive index by simulation of the optical parameters until the concentration would match is therefore possible in theory. However, in practice the variance of the calculated concentrations is too small (Figure 4-24). There is no instrument which has a sufficiently high sensitivity.

4.6.2.5 Conclusion

The results obtained from LD measurements are strongly influenced by the choice of the optical parameters applied, which was never reported to this critical extent before. Changes can influence not only the total mean size, but also the size distribution. Therefore LD measurements without the application of correct optical parameters are incorrect. Publications of LD data without the information of optical parameters used are also meaningless, as those results cannot be reproduced.

4.7 Investigations to changes of measuring conditions

Beside the computational and theoretical side, also the measuring conditions can have effects. In this part influences on the results, which might result from different measuring conditions were investigated e.g. amount of sample and time of measurement. Other conditions, e.g. temperature were never subject to investigations before. However, the refractive index is also temperature dependent; therefore this parameter was investigated. Investigations of changes in results obtained due to dissolution of the sample were also considered.

4.7.1 Impact of measuring time

The time requested for one measurement with the LS 230 depends on the mode used. It is at least 60s for one run if PIDS is included into the measurement. It is only 30s seconds if PIDS is not included and only a simple diffraction measurement is performed. Every measurement consists of at least three runs (one run gains one particle size distribution, the average of all runs/distributions performed in one row, without changing the sample (usually 3 runs), corresponds to one measurement). Therefore one complete measurement takes about three minutes per sample if PIDS is included, only 90s if analysis is performed without PIDS. Also the time needed between adding the sample to the measuring medium until the peticular measurement is started may vary. First, sample needs to be added successively until the right obscuration is reached. The time requested for this step depends on the consistency of the sample (e.g. concentration, particle size, etc.) and on the experience of the user. Second, the software requires inputs like the name of the sample, the measuring mode after the sample was added. Also here the time required changes by the velocity of the user, length of sample name, etc.

The first experimental set was performed in order to investigate changes in particle size analysis over time (i.e. time for performing the 3 runs, but also considering sample dilution time prior to start of the measurement).

Nanosuspensions show an enlarged surface in comparison to normally sized drug powders. Therefore the dissolution velocity and the saturation solubility are increased, having also an effect on poorly soluble compounds. Using the LS 230 for particle size characterisation the sample needs to be added to 135ml measuring medium, normally water. From this it is obvious that dissolution of the sample occurs to some extent during the measurement. This is also valid for poorly soluble drugs because 8-100 μ l of suspension are diluted with 135ml medium leading to dissolution effects even at very low solubilities.

The study was conducted by analysing a cyclosporine nanosuspension (batch: C5 tun 5%). PIDS analysis mode was included. Six repetitions (runs u, 1-6) have been performed. The whole procedure was repeated by using not water but saturated cyclosporine solution as measuring medium (run s 1-6). Changes in obscuration, diffraction intensity (flux values) and particle size were investigated. For particle size analysis different optical modules were used. Fraunhofer analysis was used as recommended from the handbook of the LS 230. $1.456 + 0.001i$ (* real refractive index, ** imaginary refractive index) were used as recommended from (Müller 1996) for fat emulsions. 1.49 0.03, 1.49 0 and 1.51 0.01 were used as measured real refractive indices of cyclosporine.

4.7.1.1 Changes in obscuration

The obscuration is detected from the PIDS cell (detector is located behind the PIDS cell at an angle of 180°) and from the LD cell. The obscuration values visible if sample is added to the measuring medium correspond to the obscuration values for 450 nm in case of the PIDS cell and to the 750nm (laser) for the diffraction cell. The obscuration values for the PIDS wavelengths 600nm and 900nm are detected within the measurement. They can be viewed after each run or after the whole measurement was performed. All obscuration values obtained for the cyclosporine nanosuspension analysed over six runs in unsaturated medium (= u) and over six runs in medium saturated (= s) with cyclosporine powder are shown in Figure 4-26 and in Table 4-13.

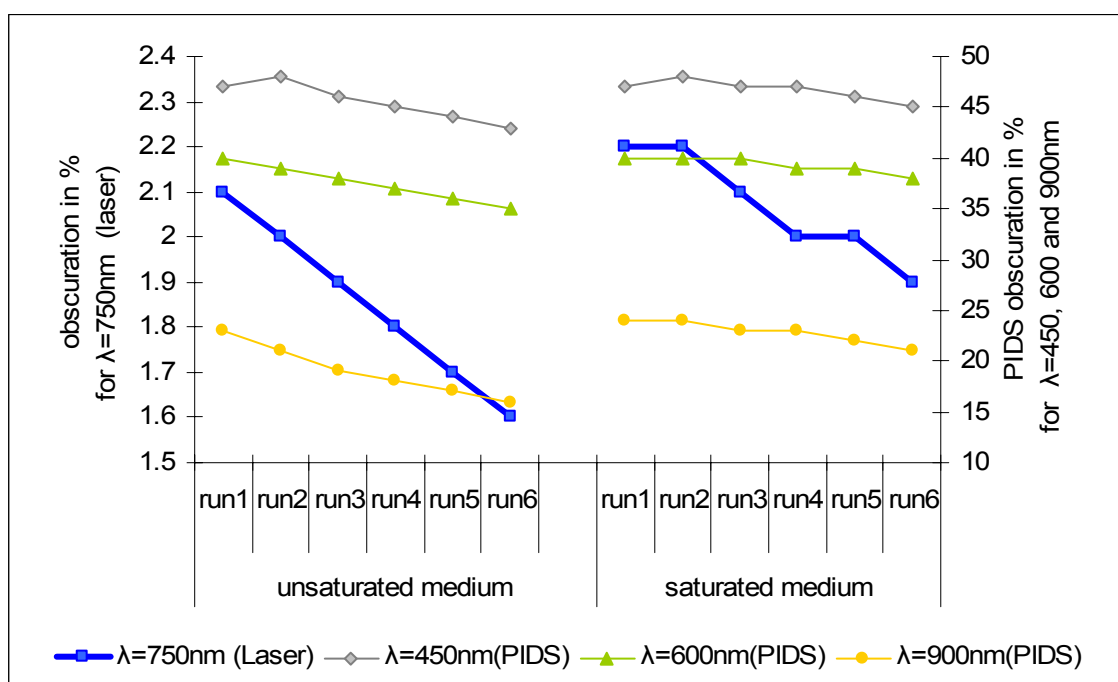


Figure 4-26: Decrease of obscuration values due to dissolution of the sample (cyclosporine nanosuspension, run 1-6 in unsaturated (= u) water and in water saturated (= s) with cyclosporine

Table 4-13: Obscuration values in % from measurements (=6 runs) of cyclosporine nanosuspension in unsaturated (upper) and saturated medium (lower), all obscuration values decrease over the time of the measurement indicating dissolution of the sample. The effect is less if medium saturated with cyclosporine powder was used.

	wavelength	run 1	run 2	run 3	run 4	run 5	run 6
unsaturated medium	750nm (laser)	2.1	2	1.9	1.8	1.7	1.6
	450nm(PIDS)	47	48	46	45	44	43
	600nm(PIDS)	40	39	38	37	36	35
	900nm(PIDS)	23	21	19	18	17	16
saturated medium	750nm (laser)	2.2	2.2	2.1	2	2	1.9
	450nm(PIDS)	47	48	47	47	46	45
	600nm(PIDS)	40	40	40	39	39	38
	900nm(PIDS)	24	24	23	23	22	21

In the unsaturated medium the obscuration decreases rapidly over the time of the measurement, indicating a loss of particles over time of the measurement, hence dissolution of the sample. Also in the saturated medium a decrease over time was obtained, indicating that the medium was not fully saturated. However, the decrease was much lower than in the unsaturated medium. In conclusion saturation of the measuring medium could decrease the dissolution process of the nanosuspension during the measurement, but could not totally avoid it.

4.7.1.2 Changes in measured intensities (flux-values)

The detected intensity in the LS 230 is measured in Flux, which corresponds to the light intensity per area and is expressed in pA (pico ampere) or mV/mm². Flux values are detected for each detector, leading to the uncorrected diffraction pattern (see 4.6.2.3). The changes of the flux values observed for the cyclosporine nanosuspension measured in unsaturated medium (= u, blue curves) and saturated medium (= s, green curves) are shown in Figure 4-27. All flux values decrease over the time of the measurement (= 6 runs). Figure 4-27 shows a decrease from one run to next run. The extent of decrease is less in the (partially) saturated medium. In Figure 4-28 the two extremes (last run from the measurement in unsaturated medium (= u run6) and the first run from the partially saturated medium (=s run1) were compared. Not only could a decrease in intensity be observed but – much more important - also a change in the obtained scattering pattern. The undissolved sample (s run1) clearly shows more minima and maxima than the dissolved sample does (u run6). In the graph it is indicated by the red arrows (Figure 4-28). The observed change will affect the calculated result, i.e. it will lead to a less accurate result as well as to an increase in the fraction of small particles, which are known to show less minima and maxima.

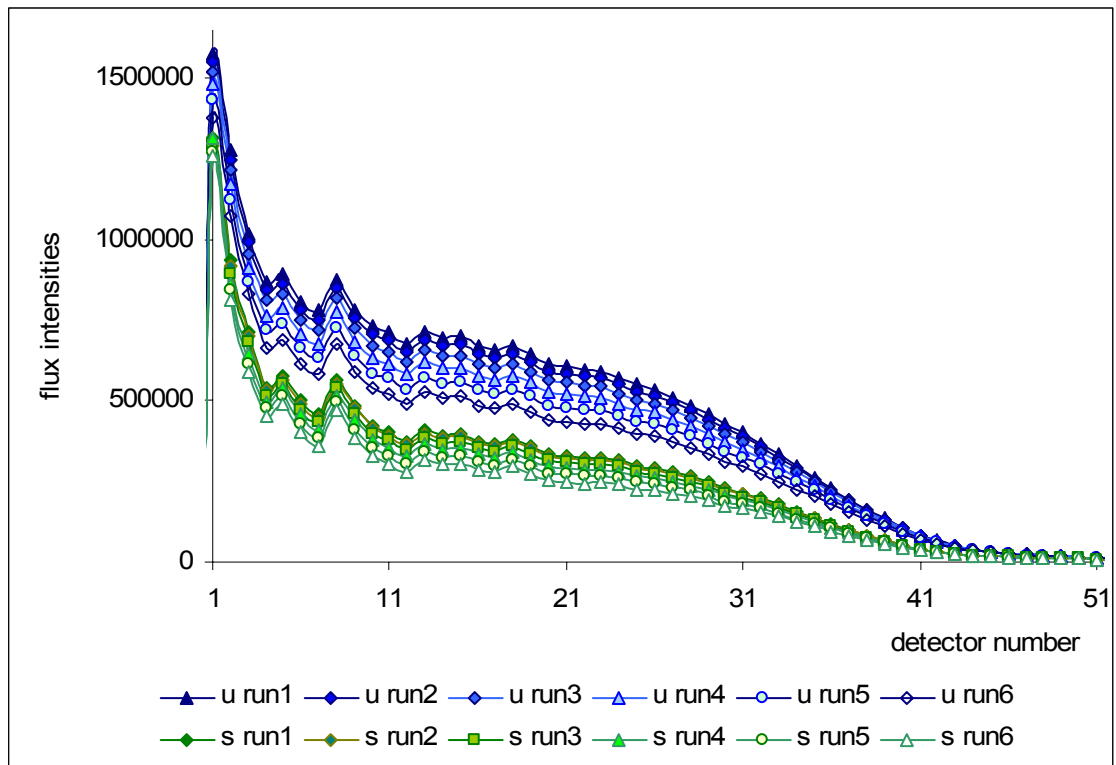


Figure 4-27: Flux intensities detected for run1 – run6 when cyclosporine nanosuspension was analysed blue-in unsaturated medium (water), green in water saturated with cyclosporine powder. The decrease in flux intensities over the time of the measurement is less if medium saturated with cyclosporine was used.

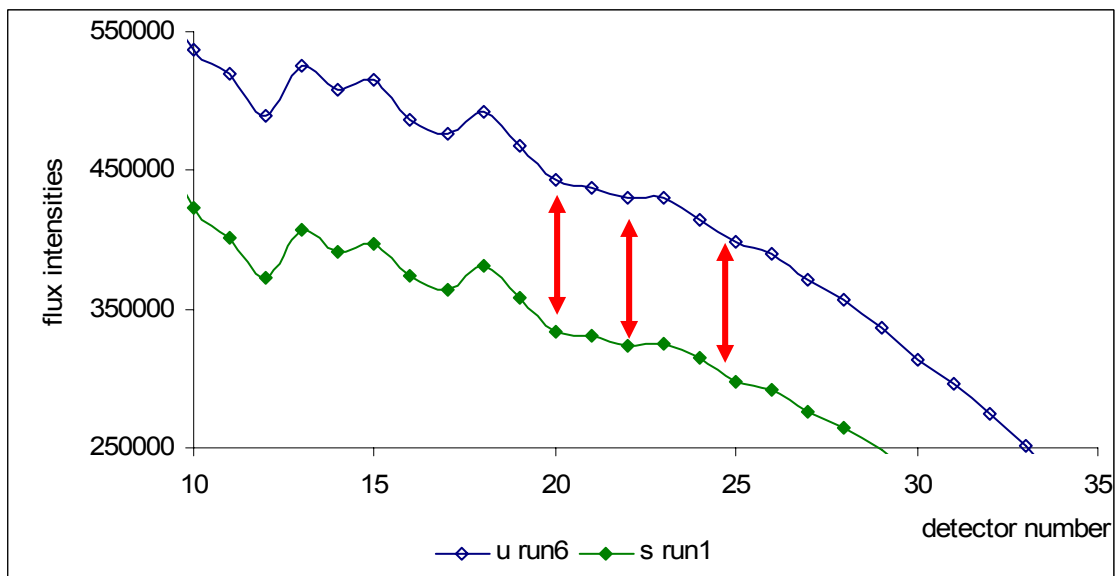


Figure 4-28: comparison of flux intensities from the first run (s run 1) in saturated medium (green) and the last run (u run 6) in unsaturated medium (blue) –shows that not only the intensity decreases if the sample dissolves, but also that the obtained pattern changes, in the pattern obtained from the measurement in saturated medium more minima and maxima can be observed. Red arrows point out the differences. Differences are little, but might lead to a different size analysis

4.7.1.3 Changes in detected PIDS intensities

Similar to the flux data from the diffraction cell ($\lambda=750\text{nm}$), also possible changes in flux intensities from the PIDS detectors were studied. As described before, PIDS measures intensities of scattered light under different angles and different wavelengths using horizontally and vertically polarised light. Only the differences between the intensities of vertically and horizontally scattered light are used for the calculation of the particle size. The PIDS intensities from the measurement in unsaturated medium (left) and saturated medium (right) are shown in Figure 4-29. The intensities were calculated by subtracting the intensities detected from horizontally polarised light from the intensities detected for vertically polarised light. This was done for every detection angle (60° , 75° , 90° , 105° and 120°) and for each wavelength, giving 3 sets for the three wavelengths (450nm, 600nm and 900nm), each with five intensities for the 5 angles of detection.

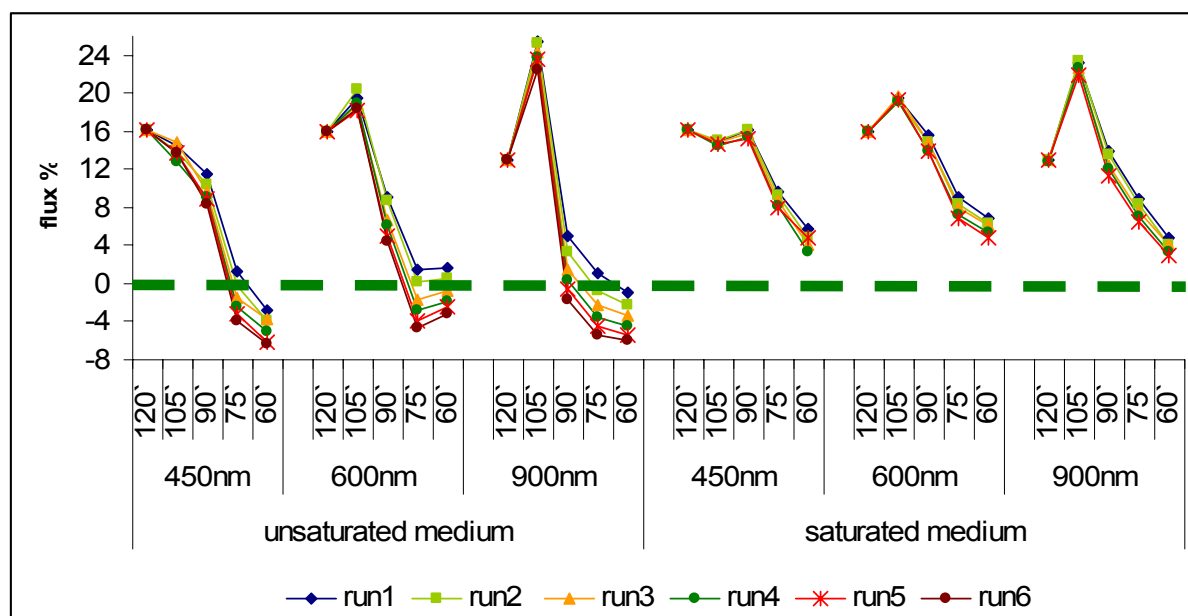


Figure 4-29: Changes in PIDS intensities obtained, for the measurement in unsaturated medium (left) and partially saturated medium (right). The PIDS intensity decreases with ongoing dissolution of the sample. The decrease is less if partially saturated medium is used. The differences (max-min) from larger to smaller angles are higher if unsaturated medium is used - indicating the presence of smaller particles, which arrive as particles become smaller if they dissolve.

As it was already seen for the diffraction patterns (in 4.7.1.2), the intensities decrease over the time of the measurement. If partially saturated medium (Figure 4-29, right) is used the changes are less when compared to the measurements in unsaturated medium (Figure 4-29, left). The changes in the flux pattern in 4.7.1.2 were little. For the PIDS data the observed effect is much more pronounced. The differences between the smallest and largest angles in the patterns from the measurement in unsaturated medium are much higher when compared to

the patterns of partially saturated medium. The higher the differences between larger and smaller angles, the smaller are the particles within the sample. Hence, the sample measured in unsaturated medium contains smaller particles than the sample measured in saturated medium. This is explained as particles dissolve over the time of the measurement and become smaller therefore. The data in Figure 4-29 also show that the difference between smallest and largest angle decreases after run 4 in case for the saturated medium. The PIDS intensity at 450 nm is higher for run 5 (red line with star, 450nm) than in run 4 (dark green line with dot). The change indicates that the small particles dissolved and only larger particles remained. In conclusion, PIDS data change similar to the diffraction intensities, but are more affected by dissolution effects. PIDS is especially sensitive to detect smaller particles or changes of them. Therefore the comparison of the raw PIDS data will give clear evidence if changes of the system occurred over the measurement.

4.7.1.4 Changes in particle sizes

In this section the calculated particle sizes from the measurements in saturated in unsaturated medium are discussed. Sizes were calculated using different optical models in order to show to impact of the optical model on the result and the interpretation of the data. Figure 4-30 shows the particle sizes obtained from the measurement in unsaturated medium. By using Fraunhofer analysis almost no changes in the mean particle size were obtained (LD 50). Only the LD 10 decreased over time. LD 90, 95, 99 and 100 are in most cases smaller when compared to the results obtained by Mie analysis.

The analysis of the data by Mie theory obviously shows a dissolution process during the measurement. First the LD 10 decreases as especially smaller particles of the sample become smaller (run1 to run 2). After the complete dissolution of the small particles the LD 10 increases (run3 and higher). At this stage of the measurement no more small particles are left and only larger particles remain. The dissolution velocity of the remaining particles is lower because of their larger size. Also the concentration gradient is decreased, as the medium is already partly saturated by the dissolved small particles, which decreases the dissolution velocity as well. With ongoing dissolution the LD 50 increases over the time whereas LD 90, 95, 99, 100 decrease.

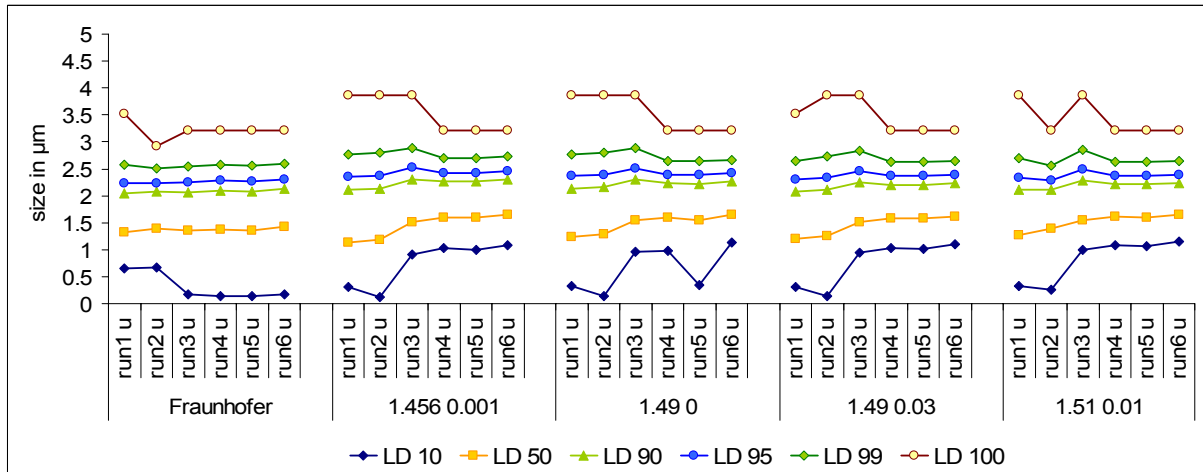


Figure 4-30: Changes in the calculated particle size distribution during a measurement (run 1-run 6) of a cyclosporine A nanosuspension, measured in unsaturated medium and analysed with different optical modules (Fraunhofer approximation, standard optical module after (Müller 1996) RI:1.456 IRI: 0.001, measured indices for cyclosporine A 1.49 RI: 1.49 and 1.51 IRI:0, 0.03 and 0.01, from left to right)

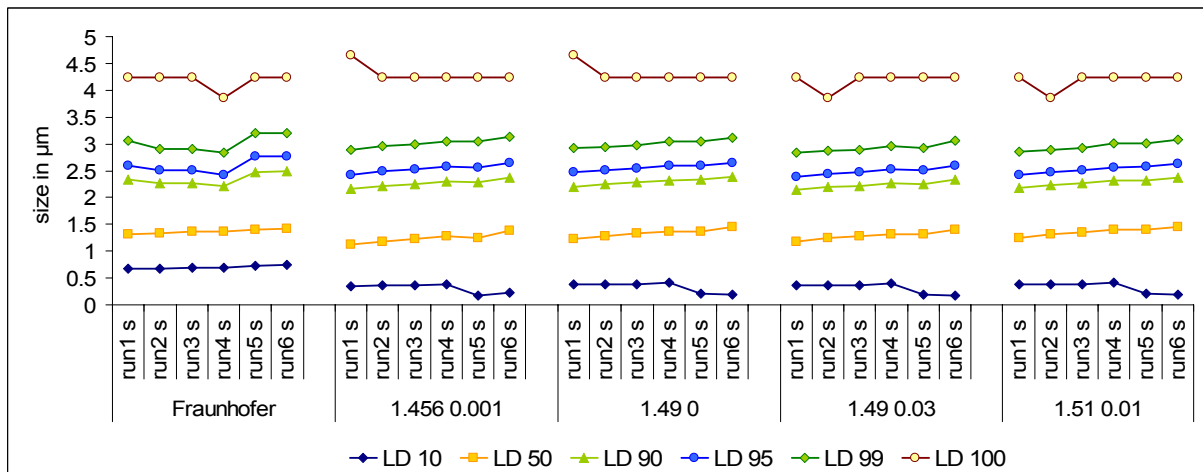


Figure 4-31: Changes in the calculated particle size distribution of a cyclosporine A nanosuspension measured in partially saturated medium and analysed with different optical modules (as in Figure 4-30)

Figure 4-31 shows an overview of the particle sizes obtained from the measurement in saturated medium. Also here the particle size changes over the time of the measurement, indicating a partially dissolution of the sample. Dissolution in this case can be explained as the saturation solubility of normally sized drug, which was used to saturate the medium, is lower than the saturation solubility of a nanosuspension. However the extend of changes due to dissolution was much lower than in the unsaturated medium. Nevertheless dissolution occurred. If the time of the measurement, especially the time passing between adding the sample and starting the measurement, is not controlled the progress of dissolution can not be estimated. Therefore the idea to use only the first run of the measurement where fewer particles are dissolved is not correct. It might be possible to use the first measurement only (as

approximation) if the time between adding the sample and starting the measurement is kept constant for each measurement. Clearly the gained result will not correspond to the real particle size of the sample, but because the time for dissolution is constant for each measurement, the results can be compared between each other to some extent.

The size characterisation of a sample is performed by using certain diameters such as the LD 10, LD 50, LD 90. The LD 50 corresponds to the mean particle size, whereas LD 10 and LD 90 are used in order to evaluate the broadness of the distribution.

Figure 4-32 gives an overview of all values obtained from the different models for the LD 50 (unsaturated measuring medium left, saturated right). The LD 50 increased when sample dissolved. Analysis by Fraunhofer did not lead to big changes in the results over time. The correlation variation (c.v.) was only 2.6%.

Using Mie theory for analysis led to clearly detectable changes in the particle sizes. This was the case for every optical model used. The c.v. was 15.7% for the model used by (Müller 1996) for nanosized emulsions and varied between 9.7% and 12.4%, when the various optical parameters for cyclosporine were used. In contrast to Mie analysis, Fraunhofer analysis of the sample measured in unsaturated medium did not detect only an increase, but also decrease in LD 50. The LD 50 first increases (run 2) and decreases again for run 3, followed by a further increase over time. In comparison to the other models, Fraunhofer LD 50 was too large for the first measurement and was too small for the last runs (unsaturated measuring medium).

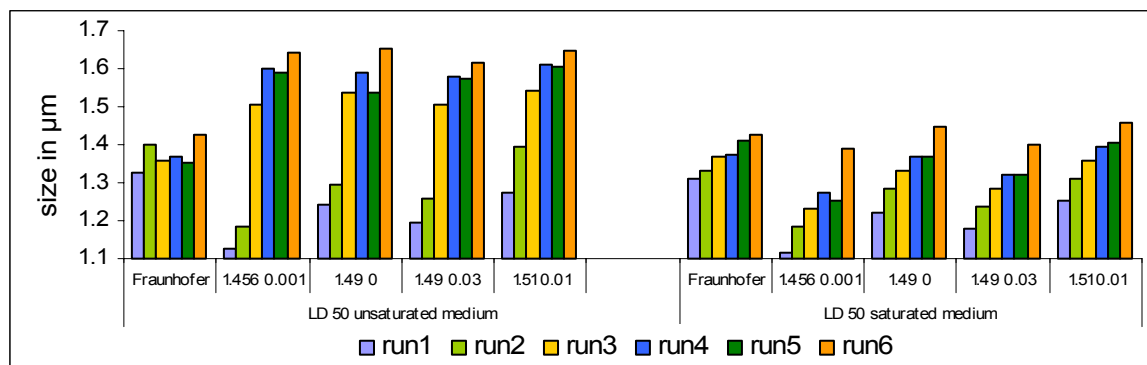


Figure 4-32: Changes of LD 50 during the measurement in unsaturated medium (left) and in partially saturated medium (right)

Figure 4-33 shows the values obtained for the LD 10. From this it is clear that especially the LD 10 is affected, if particles dissolve during the measurement. Fraunhofer analysis gives a different trend than Mie analysis. Here the LD 10 increases first and decreases with the ongoing measurement time. In Mie mode LD 10 decreases first and increases in the later measurements. Mie analysis with the optical parameters 1.49 0 detect a dramatic drop of LD 10 in the fifth run, whereas the others only detect a very little drop.

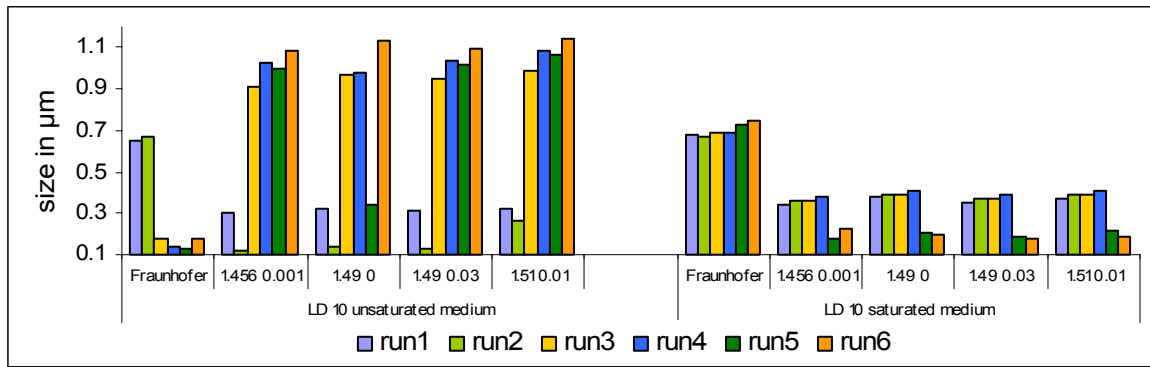


Figure 4-33: Changes of LD 10 during the measurement in unsaturated medium (left) and in partially saturated medium (right)

In Figure 4-34 the changes of the LD 90 are shown. In contrast to the changes of LD 10 and LD 50, the values of LD 90 are less influenced in the measurement in the unsaturated medium. Also the trend of change is different in unsaturated medium, when compared to the saturated medium. In unsaturated medium the LD 90 increases during the first runs and reaches a maximum after three runs. Afterwards it drops and slightly increases again. The measurement in saturated medium led to a constant increase of LD 90. But again, Fraunhofer analysis shows a totally different trend. The same trend was found for the LD 95, which is shown in Figure 4-35.

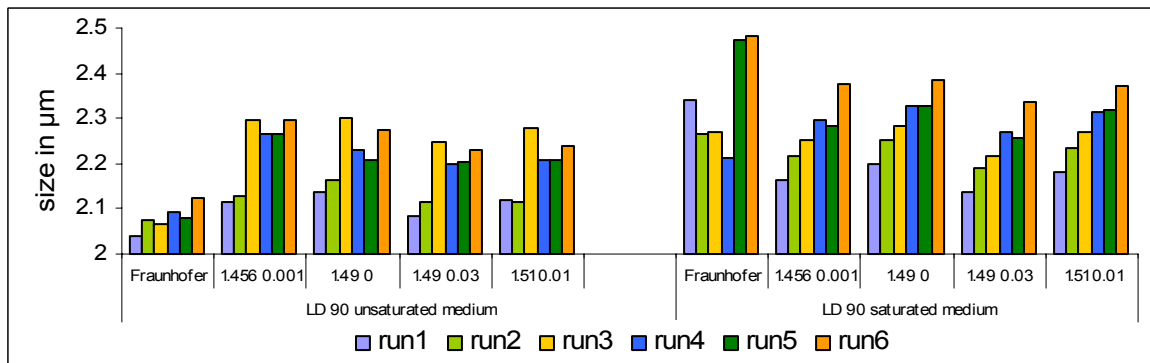


Figure 4-34: Changes of LD 90 during the measurement in unsaturated medium (left) and in partially saturated medium (right)

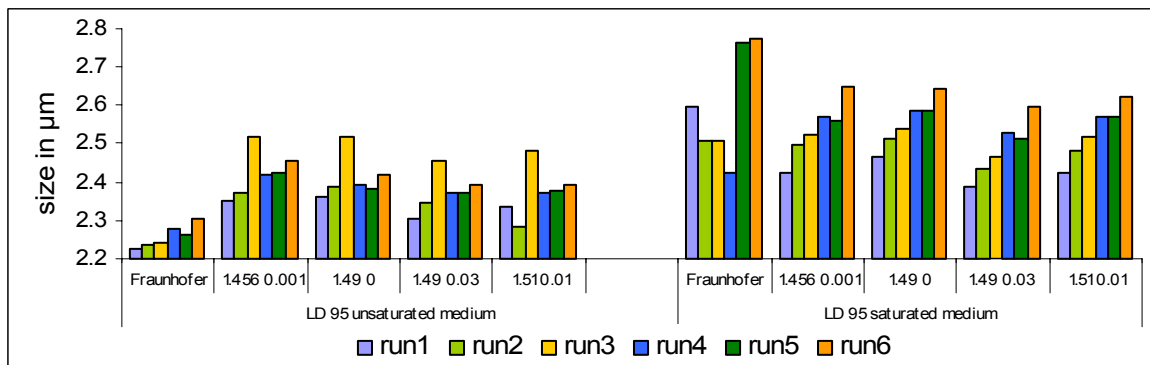


Figure 4-35: Changes of LD 95 during the measurement in unsaturated medium (left) and in partially saturated medium (right)

4.7.1.5 Conclusion

The nanosuspension dissolved during the measurement. It also dissolved during the measurement when saturated medium was used. This can be explained as the saturation solubility of normally sized drug, which was used to saturate the medium, is lower than the saturation solubility of a nanosuspension. However the extent of changes due to dissolution was much lower than in the unsaturated medium.

Particle size changes if the sample dissolves during the measurement. The extent of changes depends on the time of measurement but also on the dissolution velocity of the drug. The observed trend is an increase of LD 50 over time. LD 10 decreases first and increases after the small particles of the distribution are dissolved. LD 90 decreases slowly. The width of the distribution increases first and decreases with the ongoing dissolution process. Figure 4-36 shows the process of dissolution of small particles (1-5) and at later stage dissolution and size decrease of remaining larger particles (6-9; decrease in LD 90).

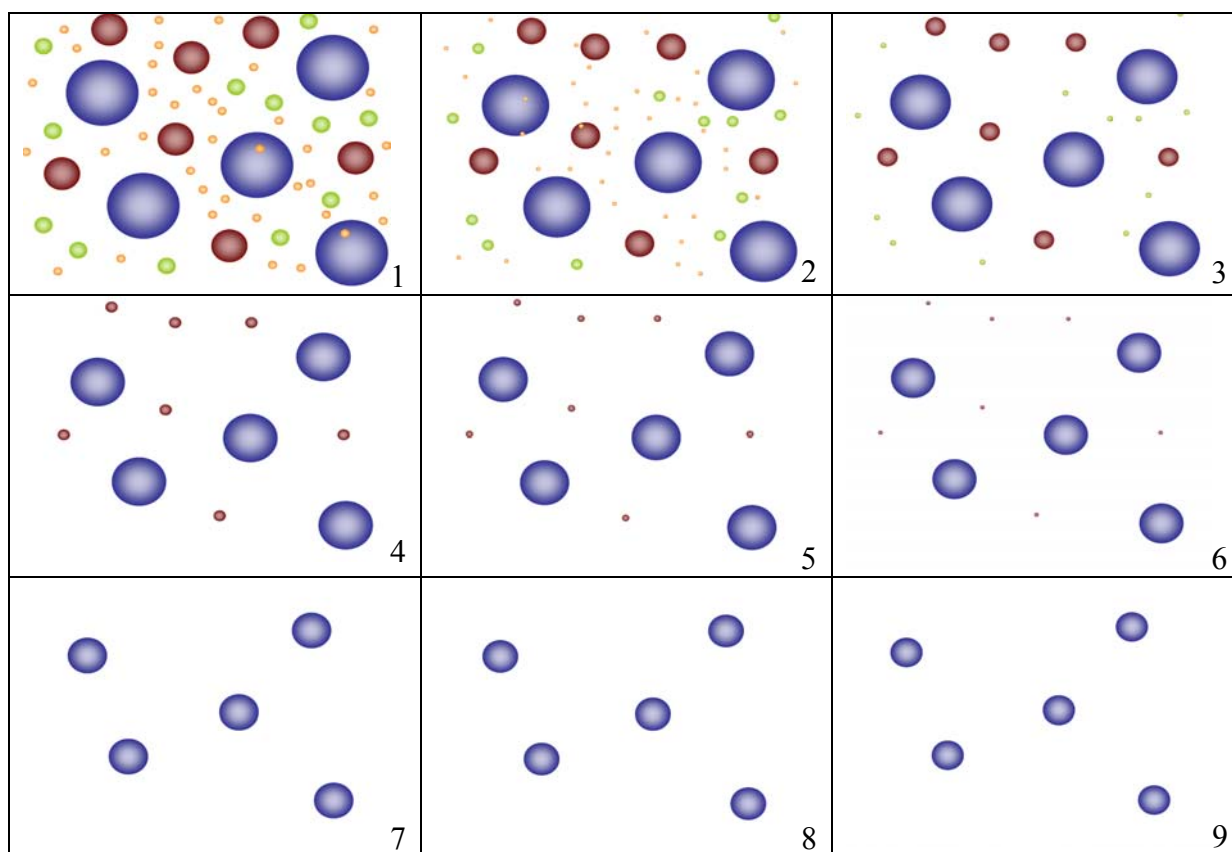


Figure 4-36: Scheme of dissolution of a nanosuspension during a measurement

From this it can be concluded; as faster a drug dissolves and/or as longer the measurement takes, as more tremendous will be the change in particle size. Therefore saturation of the medium, as well as a standardised measurement performance is recommended. It is important

to note, that also the result obtained strongly depends on the analysis mode used. Fraunhofer mode is not sensitive enough to detect changes due to dissolution of the sample. Using Mie theory with incorrect input of optical parameters can lead to non sense results. From this it is clear Mie theory can only be used, if the optical parameters are known. If a nanosuspension is analysed by not using a validated measurement procedure, the following parameters may vary from one measurement to the other:

- time required for adding the sample
- amount of added sample (how many % of sample will dissolve?, less % at larger sample amounts!)
- time required for setup of the measurement (e.g. input of sample name, optical module)
- measuring time (60-90s when PIDS is included, 30-60s if no PIDS is included)
- dissolution velocity depending on the compound, as well as on the particle size
- optical parameters

From this it is clear, that the comparison of samples, which have been analysed in a non-standardised way, is not possible, as the progress of dissolution will be different in each measurement. Performing long-term stability studies the fluctuations in the measured sizes can lead to wrong conclusions regarding suspensions stability. Perfectly stable suspensions might be judged as instable and vice versa.

For an improvement of the quality of the size measurement it is therefore suggested to use only saturated medium for the size characterisation of a nanosuspension. The software of the LS 230 allows setting up the measurement parameters, as well as entering the name of the sample, prior the adding of the sample to the measurement medium. This should be done in order to keep the time of each measurement constant. The amount of sample added should be documented and changes in obscuration should be checked and documented after every measurement as well.

4.7.2 Dissolution of cyclosporine nanosuspensions during measurements

In the previous experiment it was shown, nanosuspensions dissolve during the measurement if the medium is not saturated. It was also shown, that saturation by normally sized powder is not sufficient, as the saturation solubility of nanosuspensions is increased. It was assumed that only measurements in fully saturated medium will avoid changes in particle sizes during the measurement. Therefore a cyclosporine nanosuspension was measured not only in water (unsaturated medium) and partly saturated (powder saturated water) but also in fully saturated water. The aim of the experiment was to prove the assumption from the previous experiment. The obtained complete data set was taken to investigate the meaning of a dissolution process for the all over results in particle size characterisation by laser diffraction.

The cyclosporine nanosuspension (batch code: C3 tss d14) was measured three times using the LS 230 with Software Version 3.19. All measurements were analysed using Mie theory (real part 1.49, imaginary part 0.03). Results were analysed as volumetric (Figure 4-37) and numeric distribution (Figure 4-38).

Measurement media:

Medium 1 was pure water (us, unsaturated).

Medium 2 was saturated by cyclosporine powder with a particles size of approx 50 μ m (ps, powder saturated). 100mg cyclosporine A was added to 1000ml water. The obtained dispersion was stirred at room temperature (22.1 °C). After 24h the dispersion was filtrated. To ensure saturation of the filter used, first only 100ml of the dispersion were filtrated. The filtrate obtained was discarded.

Medium 3 (ss, super saturated) was saturated by adding the cyclosporine nanosuspension with a particle size of 650nm (z-average, PCS) to 500ml of medium 2. The dispersion was stirred at room temperature (22.1 C) for 4h and filtrated as described above.

4.7.2.1 Changes in particle size during the measurement

As described above, the nanosuspension dissolved during the measurement when the medium was not fully saturated. Changes in particles size over the time of measurement were observed in the unsaturated (us) and powder saturated medium (ps), whereas the size stayed nearly constant in the fully saturated medium (ss). Only 8 runs could be performed, when unsaturated medium was used. Afterwards the remaining particle concentration was below the detection limit of the LS 230 due to particle dissolution (Figure 4-37 and Figure 4-38).

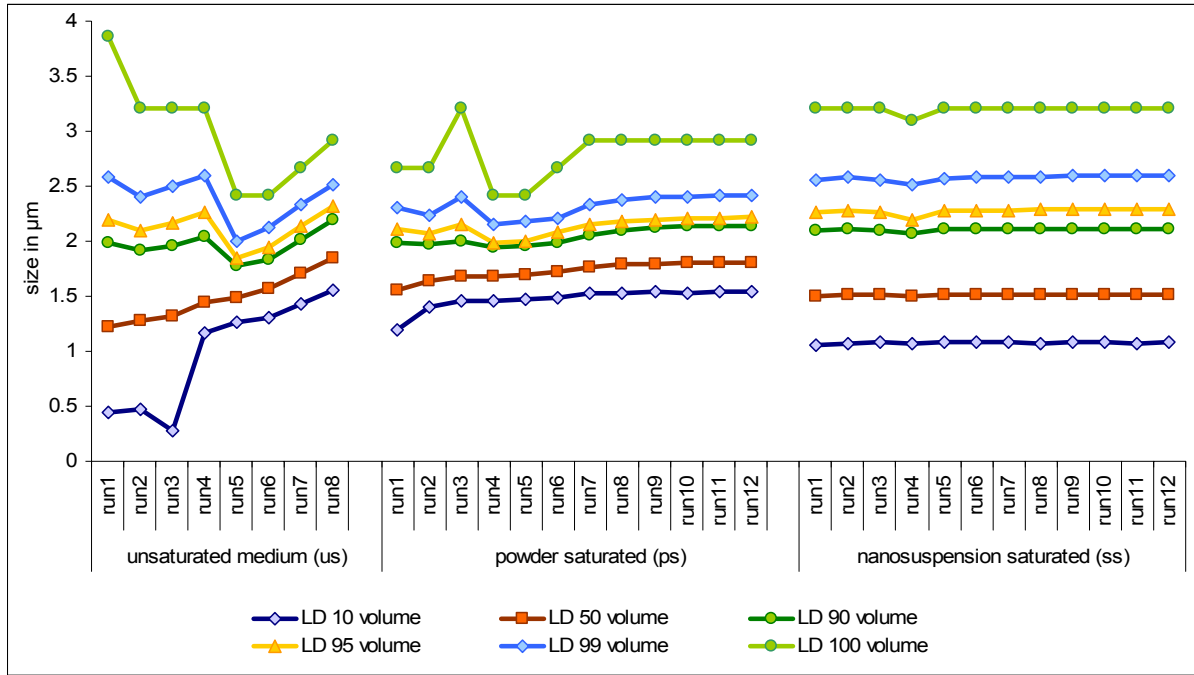


Figure 4-37: Changes in particle size due to dissolution of the nanosuspension in unsaturated medium (left), powder saturated medium (middle) and nanosuspension saturated medium (right), given are the LD diameters 10% to 100% (volumetric distribution)

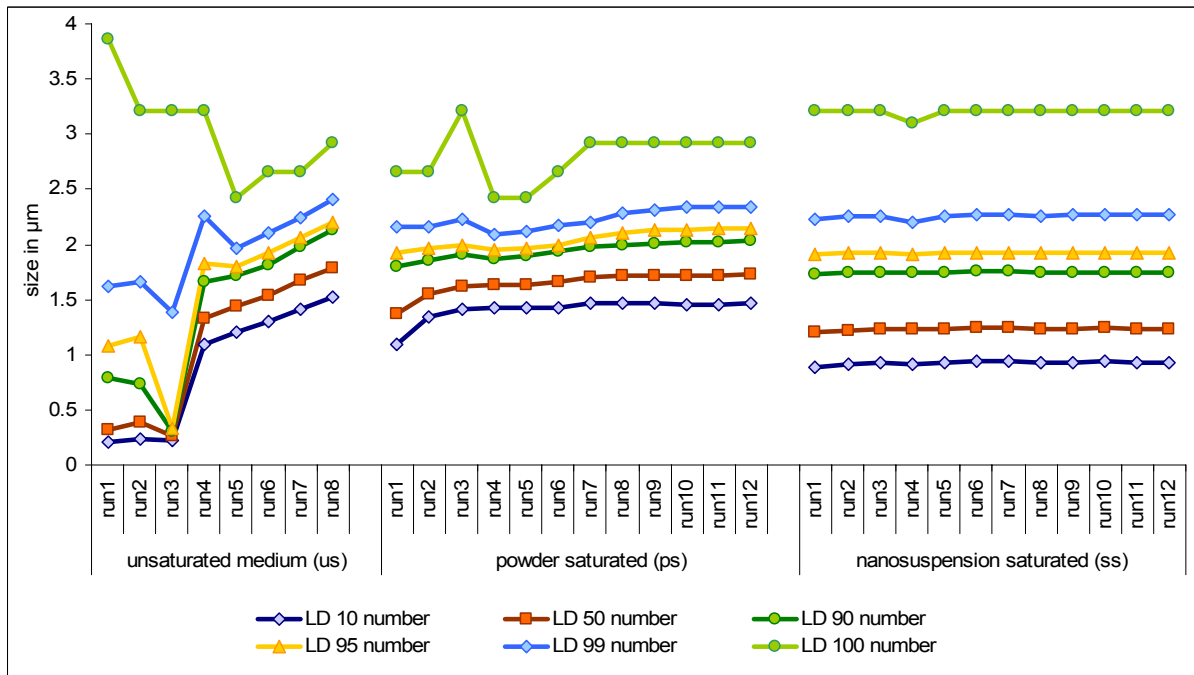


Figure 4-38: Changes in particle size due to dissolution of the nanosuspension in unsaturated medium (left), powder saturated medium (middle) and nanosuspension saturated medium (right), viewed as numeric distribution (c.f. also Figure 4-37)

The most remarkable changes in particle size occurred during the first three runs of the experiment, which corresponds to the time of one measurement, normally performed in our workgroup. Measurements normally performed include three repeated runs of 60s each and correspond to the procedure recommended by the manufacturer of the LS 230.

In order to evaluate the realistic impact of a dissolution process on the particle size obtained, only the first three runs of each measurement from this experiment were included in the analysis. It was found that in comparison to the fully saturated medium (ss) the obtained particle size (LD 50) in the unsaturated medium (us) was too small and was too large in the partially saturated medium (ps) (Figure 4-39).

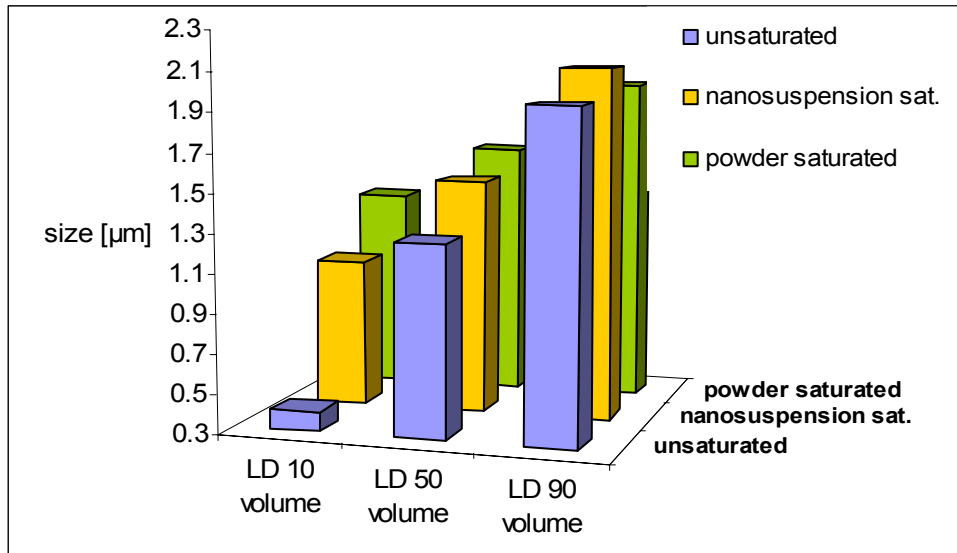


Figure 4-39: Differences in particle size due to dissolution of the nanosuspension during a standard measurement (3 runs a 60s) in unsaturated medium (front), powder saturated medium (middle) and nanosuspension saturated medium (back), viewed as volumetric distribution

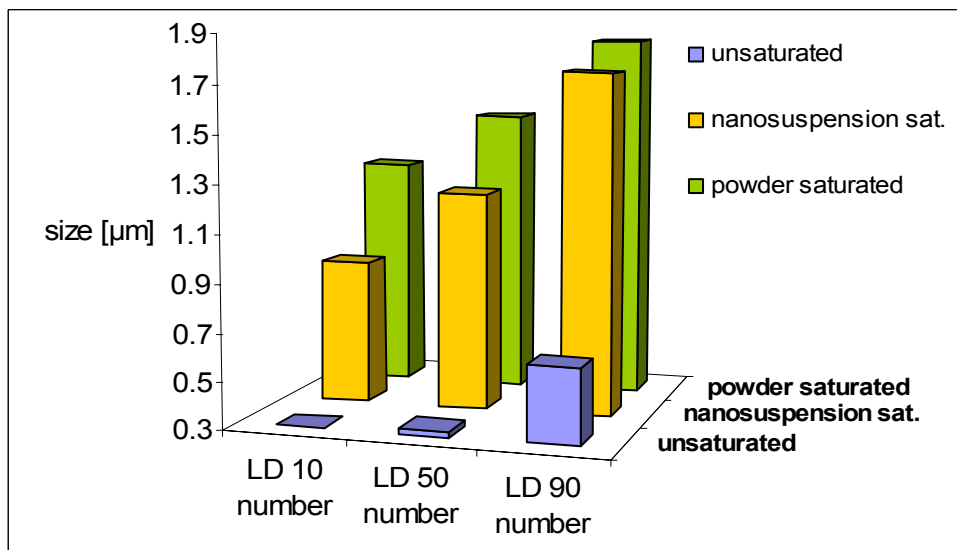


Figure 4-40: Differences in particle size due to dissolution of the nanosuspension during a standard measurement (3 runs a 60s) in unsaturated medium (front), powder saturated medium (middle) and nanosuspension saturated medium (back), viewed as numeric distribution

The particle size distribution was found to be too narrow in the partially saturated medium and too broad in the unsaturated medium (Figure 4-41).

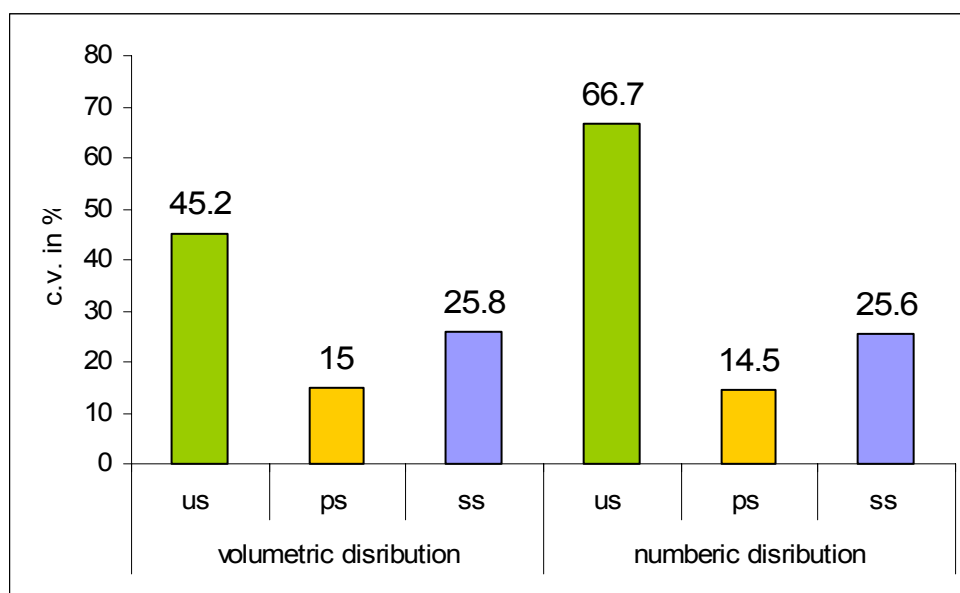


Figure 4-41: Correlation variation in particle size due to dissolution of the nanosuspension during a standard measurement (3 runs a 60s) in unsaturated medium (left), powder saturated medium (middle) and nanosuspension saturated medium (right), viewed as volumetric (left side) and numeric distribution (right side)

The most affected value was the LD 10. This can be explained by the fact that especially the small particles dissolve faster than larger ones. The extremely small LD 10 values in case of the unsaturated medium (us) can be explained, as larger particles become smaller over time until saturation equilibrium is reached, which can also be seen in a decrease of LD 50 and LD 90. If partially saturated medium is used only very small particles dissolve, whereas larger particles do not. This is because their saturation equilibrium is already reached. At the end this causes the too large particle size distribution and the narrower width of the size distribution obtained. Also it is important to realise, that LD 90, 95, 99 and 100 are smaller when not fully saturated medium was used. This indicates that larger particles cannot be detected if the concentration of small particles is too high.

In the last few years there is a trend to publish data obtained by laser diffraction not as a volumetric but as a numeric distribution. In contrast to the volumetric distribution, where the volume of the particle is taken into account for analysis, the numeric distribution takes the number of particles into account. For example, a dispersion consists of 1001 cubic particles. 1000 of the particles show a diameter of $1\mu\text{m}$, only 1 particle has a diameter of $10\mu\text{m}$. The corresponding total volume of the particles would be $2000\mu\text{m}^3$. $1000\mu\text{m}^3$ are contributed by the small particles, $1000\mu\text{m}^3$ correspond to the volume of the single $10\mu\text{m}$ particle. In volumetric analysis the mean volume is 2000:1001 particles, i.e. approximately $2\mu\text{m}^3$. The volumetric LD 50 is the 3rd square route, i.e. approximately $1.25\mu\text{m}$. In numeric analysis this

corresponds to an LD 50 of 1.00 μm , whereas the single 10 μm particle becomes neglectable beside the 1000 small particles in the numeric distribution. Therefore numeric analysis always leads to smaller analysis results, especially when polydisperse samples are analysed, which seems to be more attractive when publishing data. To be able to judge the effect of a wrong LD measurement on numeric data, additionally the above data were analysed as numeric distribution. Results from this analysis are even much more influenced by the dissolution process than in the volumetric analysis (Figure 4-42).

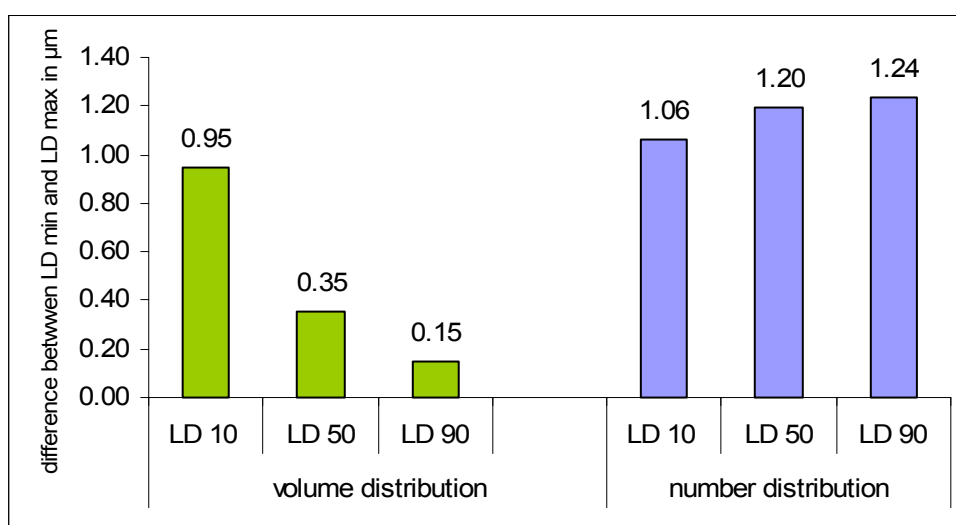


Figure 4-42: Differences between maximal values and minimal LD values (in μm) of a cyclosporine nanosuspension measured in saturated, partially and unsaturated medium (volume distribution (left) and number distribution (right)); the differences in the particle sizes calculated are less in the volume distribution. The LD 10 is the most influenced parameter in the volume distribution when changing medium. In the number distribution the LD 90 is the most influenced parameter (c.f. Table 4-14)

Table 4-14: Overview of changes in LD values due to dissolution of the nanosuspension

	measuring medium	LD 10 in μm	LD 50 in μm	LD 90 in μm
volume distribution	unsaturated	0.397	1.273	1.954
	powder saturated	1.347	1.624	1.986
	nanosuspension sat.	1.065	1.510	2.104
	difference (max-min)	0.950	0.351	0.150
number distribution	unsaturated	0.222	0.320	0.613
	powder saturated	1.282	1.515	1.852
	nanosuspension sat.	0.906	1.220	1.738
	difference (max-min)	1.060	1.195	1.239

In opposite to the volumetric distribution, the LD 90 and not the LD 10 is the most affected parameter, as the number and not the volume of the particles is taken into account.

In Table 4-14 the results obtained from the different analyses are listed for a direct comparison. The LD 50 is often used as the mean parameter for particle size characterisation. It shows a difference of 237nm (-15.7%) between the measurement in the unsaturated and the

nanosuspension measured in fully saturated medium in the volume distribution, but it shows the huge difference of 900nm (-78.85!), when the measurements were analysed as a numeric distribution.

In the results discussed above, only 12 runs were analysed for partially saturated (ps) and supersaturated (ss) dispersion media (please note; only 8 runs could be performed in unsaturated water (us) due to nanocrystal solution). When the time of the measurement was prolonged to 15 runs, there was an increase in LD 99 and LD 100 for the measurement in fully saturated medium (ss) (Figure 4-43). The experiment was repeated several times, to exclude a mistake in conducting the measurement procedure or the software, but the increase in particle size after a certain time was reproducible. The reason for this can be agglomeration of the sample, leading to larger particles.

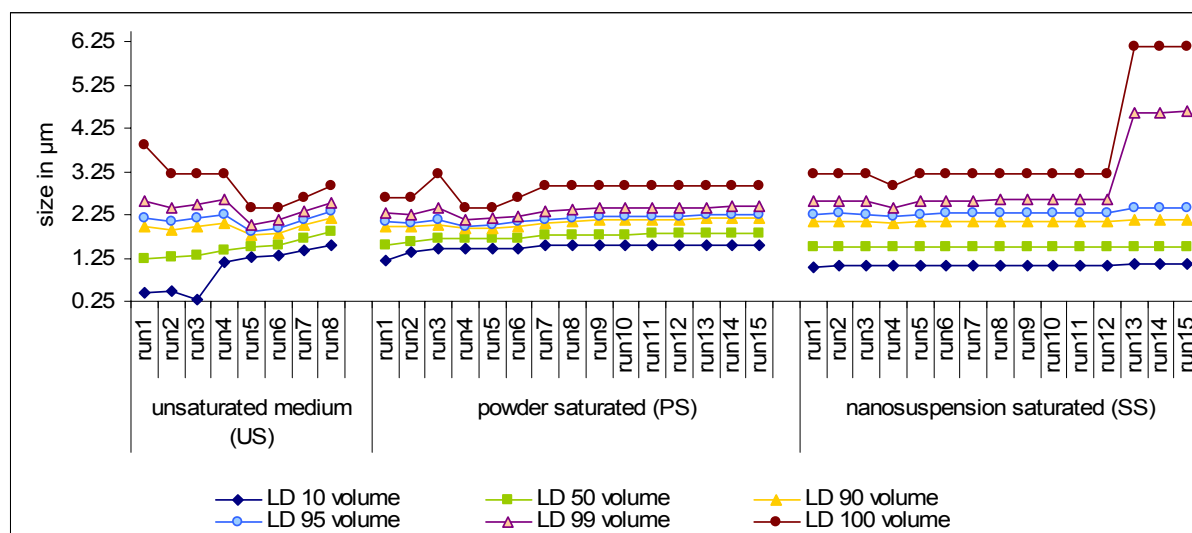


Figure 4-43: Dissolution of cyclosporine nanosuspension, longer measurements with 15 runs in super saturated medium caused an increase in LD 99 and 100 after 12 measurements

However, to prove this assumption, the diluted sample was collected after the measurement and was analysed by light microscopy. To increase to amount of particles collected from the instrument for microscopy, the collected sample was rested without motion for 2 hours, for sedimentation of larger particles. The picture obtained from that clearly shows no agglomerated particles, but larger crystals (Figure 4-44). From this it is concluded, that a cyclosporine nanosuspensions can re-crystallise during a measurement, if fully saturated medium is used!

From the literature it is known, that cyclosporine is inversingly soluble to the temperature; hence the saturation solubility decreases with an increase in temperature. Therefore the only reason for the observation could be an increase in temperature during a measurement. Therefore changes in temperature during the measurement were investigated.

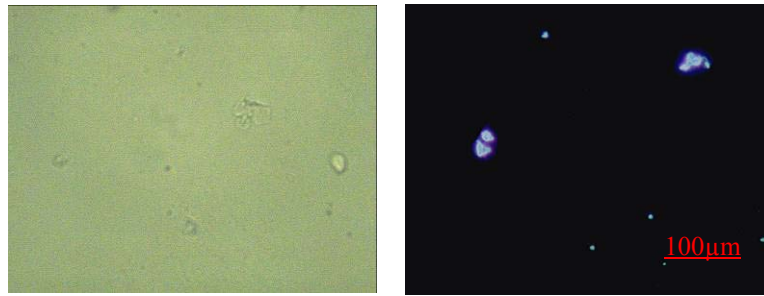


Figure 4-44: Cyclosporine crystals created during LD measurements in supersaturated media (left: non polarised light; right: polarised light; magnification 160x)

4.7.2.2 Changes in temperature during the measurement

The medium used for a measurement is normally purified water. The water is collected from the tap and e.g. stored in a 20l storage container for usage of the instrument. Three measurements were performed. The first measurement was carried out by using freshly obtained water from the tap. The temperature was 22.1°C. The second measurement was performed 5h later, using the same water, stored at room temperature in between, having a temperature of 23.2°C. The third measurement was undertaken 2 hours later, the temperature increase was only 0.1°C. In each measurement the temperature of the water was monitored during the different steps of the measuring procedure, which means after:

- degassing the water by stirring at different speeds in the cell (de-bubbling process)
- alignment of the laser prior the measurement
- performing the background measurement
- addition of sample and
- performed runs (no.1, 3, 5, 10, 20 and 30)

All measurements of the temperature were performed with a digital thermometer IKA-TRON DTM 10 (Janke & Kunkel, Staufen, Germany)

The results are shown in Figure 4-45. The increase in temperature during the measurement was unexpectedly high. The temperature increased almost 10°C within the measurement time, and reached temperatures above 30°C. These findings are relevant not only for cyclosporine nanosuspensions, but also in respect to the temperature dependency of the refractive index, required for the measurements of submicron particles. Therefore further studies were performed on this subject (see 4.7.5).

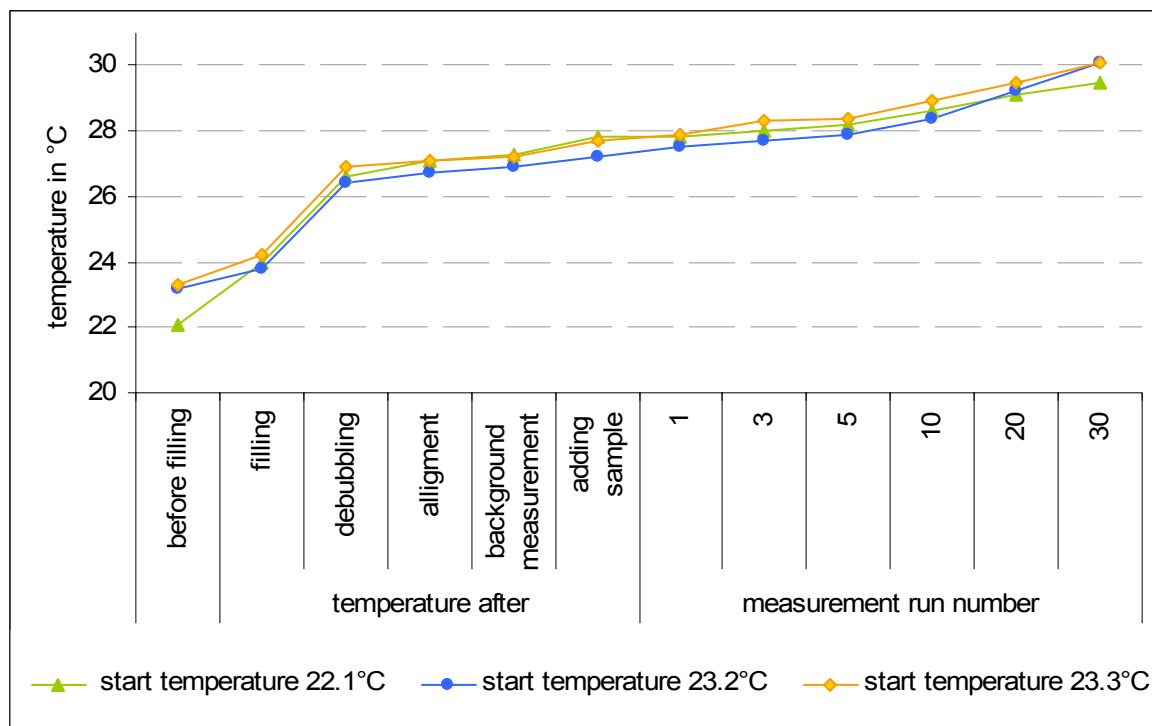


Figure 4-45: Changes in temperature of the measuring media during LD measurement

4.7.2.3 Conclusion

Only fully saturated dilution medium avoids dissolution of nanocrystals. In case of nanocrystals, the saturated dilution medium needs to be saturated using nanocrystals, because they possess a higher saturation solubility than normally sized drug powders.

Using a numeric analysis mode instead of the volumetric mode can lead to a total misinterpretation of the results if the medium is not fully saturated. Therefore the numeric mode should never be used.

All over it is concluded, that accurate particle sizing by laser diffractometry is very challenging in case of drug nanocrystals or other partially dissolving material. Only a carefully validated measurement procedure will lead to reproducible and reliable results.

4.7.3 Detection of larger particles beside a small sized bulk population

In the previous studies there was some evidence, that the detection of larger particles might be problematic when a small sized bulk population is present. However one of the aims of laser diffractometry is the detection of those particles. Failure in detection is crucial for many applications.

4.7.3.1 Influence on measuring mode used

Using the LS 230 for particle size characterisation gives the opportunity to analyse particles in two different analyses modi. PIDS technology can be applied, leading to a combined result of scattering pattern analysis and diffraction analyses. Also it is possible to exclude PIDS, than a sole diffraction analysis is obtained. Measurements without PIDS can analyse particles with sizes ranging from 400nm-2000µm. If PIDS is included in the measurement particles from 40nm-2000µm can be analysed. Therefore nanosuspensions and solid lipid nanoparticles (SLN) or nanostructured lipid carriers (NLC) dispersions were typically analysed by including PIDS, because of there small sizes. The aim of this experiment was to investigate the sensitivity of the two methods for the detection of larger particles. Therefore a model sample, containing larger particles beside a small bulk population was produced and analysed by both modi. The model chosen was an overloaded NLC formulation containing tretinoine (see Table 4-15). If NLC contain drug well above its saturation solubility in the solid lipid phase, expulsion of the drug from the lipid particles can occur, leading to formation growth drug crystals and their subsequent growth in the water phase. Therefore crystal formation and growth of drug crystals over the time in the water phase was expected for the model NLC suspension chosen (Table 4-15). The NLC formulation was produced by high pressure homogenisation applying 3 cycles at 500bar. LD analysis was performed at day 28 (d28) after production. Light microscopy was applied as additional technique to control the LD results obtained.

Table 4-15: Tretinoine NLC formulation

stearyl alcohol	17.73%
Miglyol 812	1.97%
tretinoine	0.30%
Tween 80	2.00%
water ad	100.00%

4.7.3.2 Differences in particle sizes by variation of the characterisation method

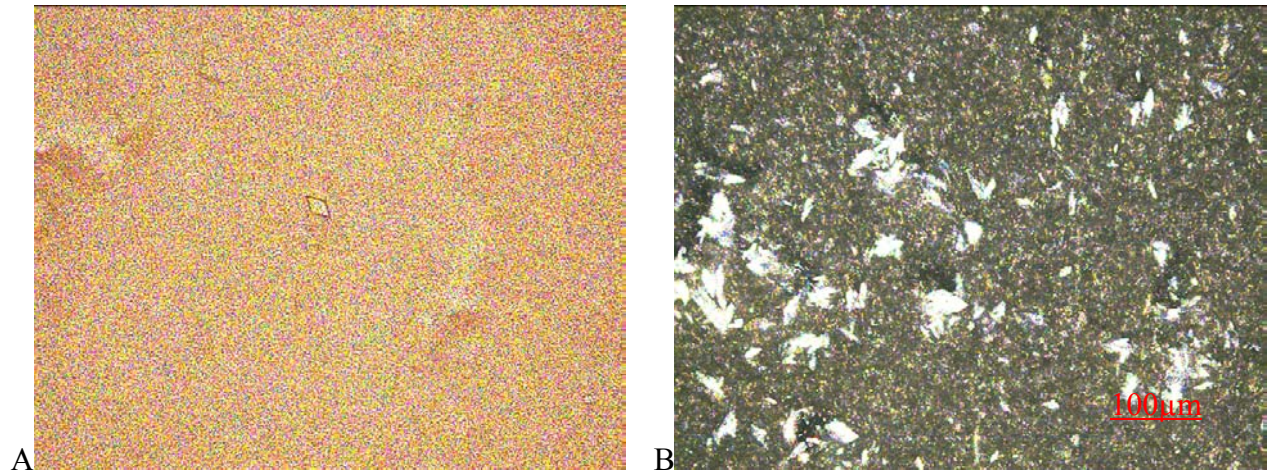


Figure 4-46: Microscopic images at day 28 of the tretinoine NLC Formulation (A without polarisation filter, B by applying polarised light (magnifications 160x))

In Figure 4-46 the microscopic images of the tretinoine formulation at day 28 are shown. Image A and B show the same object with identical magnification (160x). Image A was obtained with non-polarised light. The sample appears small sized and homogeneous, only one larger crystal (35µm) was detected. Crystals could not be seen because NLC adhered to the crystal surface, making them extremely difficult to see. In contrast is the image B. The observation of the sample by using polarised light clearly shows the existence of large tretinoine crystals within the whole sample.

The data obtained from the LD measurements including PIDS are shown in Figure 4-47. To exclude errors due to incorrect optical parameters, the raw data recorded were simulated by using a broad range of possible optical parameters (imaginary values ranging from 0-1, real indices varying from 1.3-1.8), giving a total of 60 possible results.

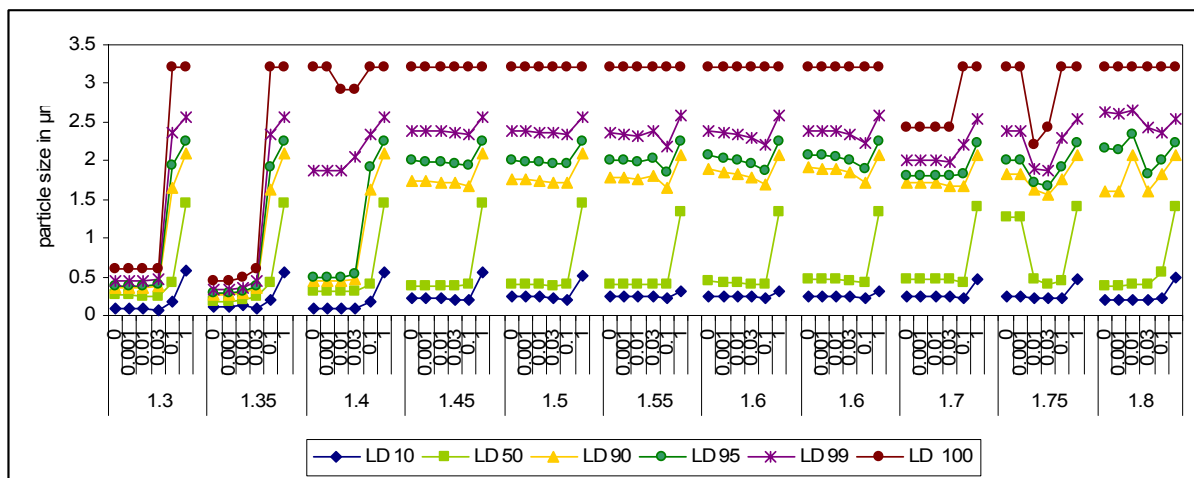


Figure 4-47: LD data for tretinoine NLC formulation with included PIDS

The LD measurement by including PIDS into the measurement set up did not detect crystals larger than 3.5 μm . In contrast LD measurements with excluded PIDS (Figure 4-48), where large particles were observed for all results obtained from the simulations. Figure 4-49 shows the calculated distributions curves for the measurements with and without PIDS. The curves were calculated with Mie mode using the optical parameters which were determined for the system (1.51 (real refractive index) and 0.003 (imaginary refractive index) (c.f. 5.3.).

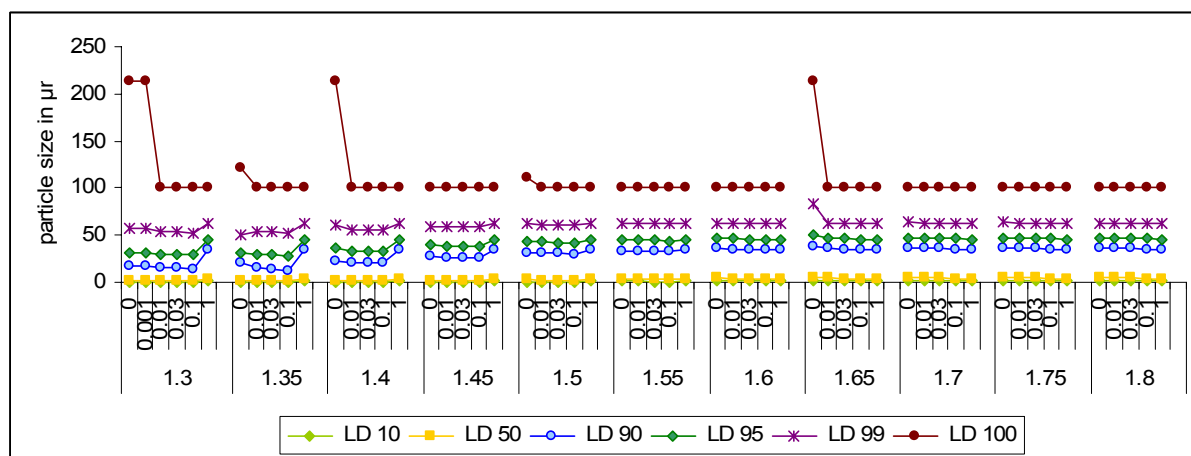


Figure 4-48: LD data for tretinoine NLC formulation without PIDS

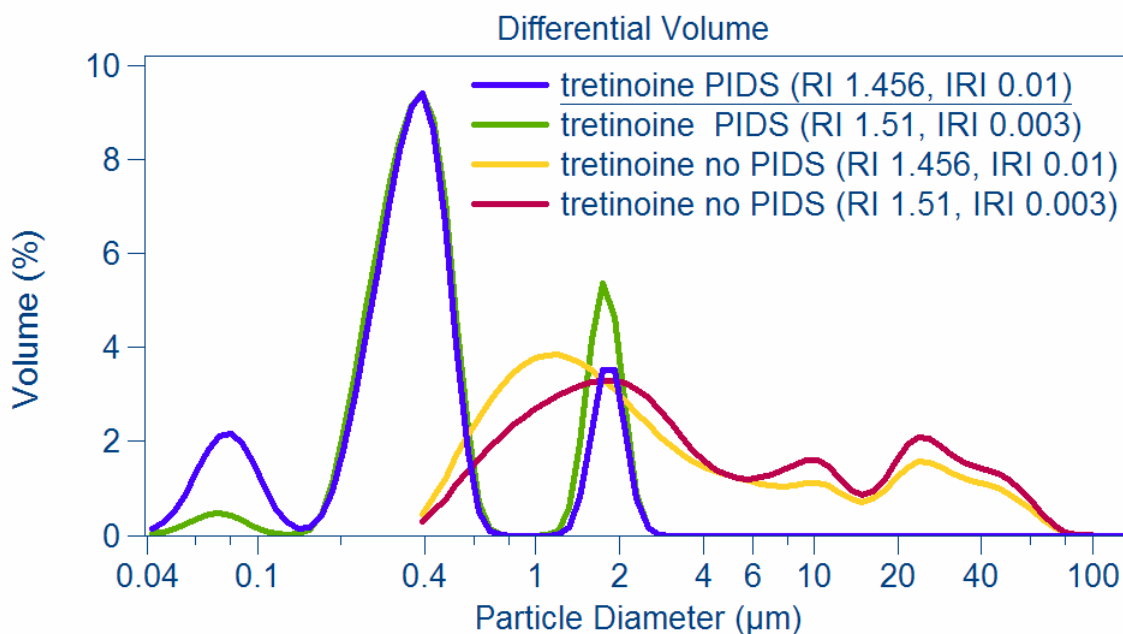


Figure 4-49: Overlay of results obtained from LD measurements with PIDS (green and blue) and without PIDS (orange and red) calculated using Mie mode (optical parameters were used after (Müller 1996) (1.456 (RI) and 0,01 (IRI) and as analysed for the system; 1.51 (RI) and 0.03 (IRI) (c.f. 5.3.).

According to Muller and Schumann, for o/w emulsions the real refractive index is 1.465 and the imaginary refractive index is 0.01. O/w emulsions are similar in composition to SLN/NLC in respect to the main lipid component. However the optical properties vary, dependent on the

specific kind of lipid used and on the drug and drug amount incorporated into the SLN or NLC. Figure 4-49 shows the distribution curves for the measurements of the tretinoine NLC formulation with and without PIDS. The results were calculated using Mie mode with the optical parameters for the fat emulsions (RI 1.456; IRI 0.01) and with the determined refractive index for the tretinoine formulation (RI 1.51; IRI 0.003; c.f. 5.3.). From this the distribution curves are relatively similar, that means by excluding PIDS the larger particles were also detected using the indices suggested by (Müller 1996) for intravenous fat emulsions. The LD data without PIDS from Figure 4-49 are shown again as diagrams in Figure 4-50. Even though the LD 100 is identical for both calculations with different refractive indices, the LD 50 as a measure for the mean particle size varies tremendous. It is 1.96 μm for the optical parameter suggested by (Müller 1996) but 2.81 μm for the correct optical parameters. From this it is concluded that the detection of larger particles is relatively independent from the optical modules used (c.f. Figure 4-48) if PIDS is excluded from the measurement. But the correct distribution can only be calculated if the correct optical parameters are used. It was not possible to obtain the correct size distribution for the tretinoine formulation if the optical parameters for the fat emulsions were used.

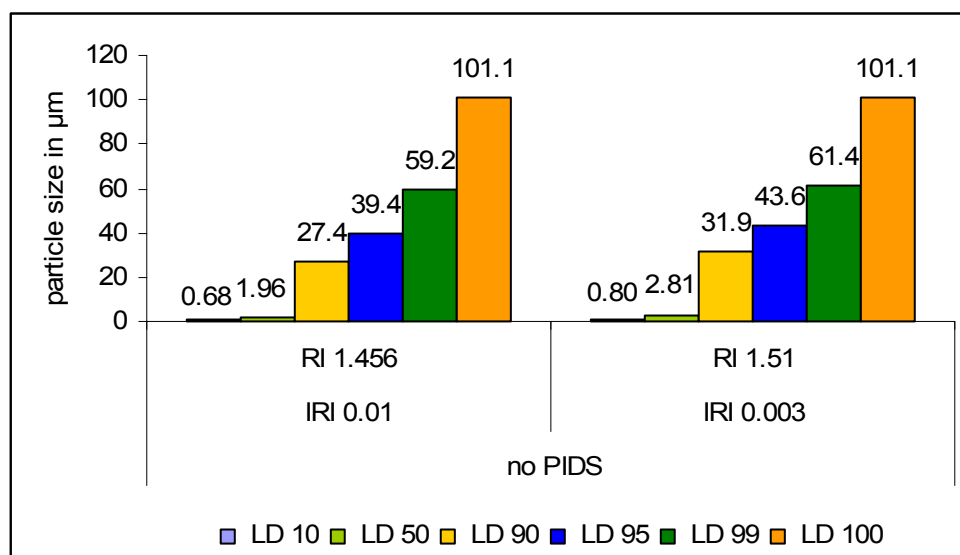


Figure 4-50: LD results obtained from LD measurements without PIDS calculated using Mie mode (optical parameters were used after (Müller 1996) (1.456 (RI) and 0,01 (IRI) and as analysed for the system; 1.51 (RI) and 0.03 (IRI) (c.f. 5.3.) Larger particles are only detected without PIDS independent if the correct index of refraction was used or only an approximation (1.456 (RI) 0.01 (IRI), for the measurements without PIDS the LD 50 is 1.96 μm for the approximate refractive index and 2.81 μm for the correct refractive index clarifying the importance of the refractive index for the correct size analysis not only for measurement with PIDS but also for measurements without PIDS

4.7.3.3 Conclusion

Light microscopy with non polarised light could not detect tretinoine crystals as NLC adhered to the surface of the crystals. The system seemed to be unchanged and stable. Tretinoine crystals show anisotropic behaviour. Therefore crystals can easily be observed under polarised light. When polarised light was applied for this analysis large crystals ranging from 40-80 μm , as well as smaller crystals ranging from 2-15 μm , became visible, clearly showing an instable and polydisperse system - as expected from theory.

Laser diffractometry with the use of the PIDS technology could not detect these large crystals, even not when the correct index of refraction was used. Measurements without PIDS technology led to the finding of large particles, similar in the size found by microscopy under polarised light. Unfortunately no small particles can be observed in this modus. Therefore the LS 230 could not correctly characterise the system. More over from the results obtained here, it is evident, that the characterisation of systems containing large crystals beside a small sized bulk population is not correctly performed by including PIDS in the LD measurement. Large particles are overseen by this method, leading to a fatal misinterpretation of the system analysed.

4.7.4 Influence of sample drawing and sampling position

Another subject to study was the influence on the sampling. If nanosuspensions are physically instable agglomeration and/or crystal growth occurs over the time of storage. Sedimentation therefore takes place, leading to an inhomogeneous distribution of particles within the suspension. Emulsions usually show floatation effects. Drug loaded NLC can show both effects, as drug can crystallise in the water phase and sediment, whereas fat agglomerates would float. Therefore, the position from where the sample is collected for further particle size characterisation is important (Muller and Heinemann 1991). However, in the previous study it was shown that LD measurements with included PIDS failed to detect large particles, whereas particles were detected if PIDS was not included in the measurement. Unfortunately the amount of sample required for measurements without PIDS is about 10-20 times higher as for measurements with included PIDS. The production of nanosuspensions and NLC using an APV 40 only yields a total 40ml. Thus, the volumes required for characterisation issues must be kept low. Measurements without PIDS may require amounts e.g. 2ml, making this method inconvenient for the characterisation of nanosuspensions and NLC formulations. The aim of this study therefore was to investigate alternative possibilities for correct particle sizing, e.g. sampling from different positions. It was expected to obtain different results for samples collected from the bottom or top, where larger particles are present in higher concentrations

than from samples collected from the middle of the vial or after shaking the vial. If LD measurements with included PIDS could reliably detect large particles from samples which were collected from the top or bottom, the amount of sample required for the size characterisation in stability analysis procedure would be much smaller and one could save valuable sample. For the study an obviously instable and inhomogeneous solid lipid nanoparticle dispersion was chosen (Stab. 3).

Table 4-16: Formulation of the SLN formulation (batch code: Stab. 3)

cetylpalmitate	15.00%
Miglyol 812	5.00%
Tween 80	1.80%
water ad	100.00%

The macroscopic analysis already showed agglomerates and larger particles within the vial. Larger particles were visible on the bottom of the vial as well as on the surface of the sample, indicating an inhomogeneous distribution of particle sizes within the sample. The vial, containing the sample (approx. 30ml) was left without any motion for 24h at room temperature. Three samples a 1ml were collected by using a syringe (2ml) and a needle (60x1mm). The first sample was carefully collected from the surface of the dispersion. The second sample was collected from the centre of the vial and the third sample was obtained from the bottom of the vial. Between the samplings the dispersion was rested for 30min in order to minimise the disturbance of particles due to the previous sampling. A fourth sample was obtained after the whole dispersion was shaken for 30s. All probes were analysed by laser diffractometry using the LS 230. Each sample was analysed with PIDS and without PIDS technology at the ideal concentration for each mode. Each measurement included three single runs. Each measurement was repeated three times. For each sample all together 18 measurements were performed. Measuring conditions were kept constant. The obscuration for measurements including PIDS technology was 42-48% and between 7-9% for the measurements without PIDS. The measurement time for PIDS included measurements was 60s and 30s for measurements without PIDS. All results were compared in order to investigate the reliability of measurements as well as to optimise the reliability of analysis in respect of the detection of larger particles.

Analysis was performed both with Mie and Fraunhofer theory, resulting in four different combinations

- PIDS with Mie
- PIDS with Fraunhofer
- no PIDS, Mie only
- no PIDS, Fraunhofer only

4.7.4.1 LD-measurements with and without PIDS

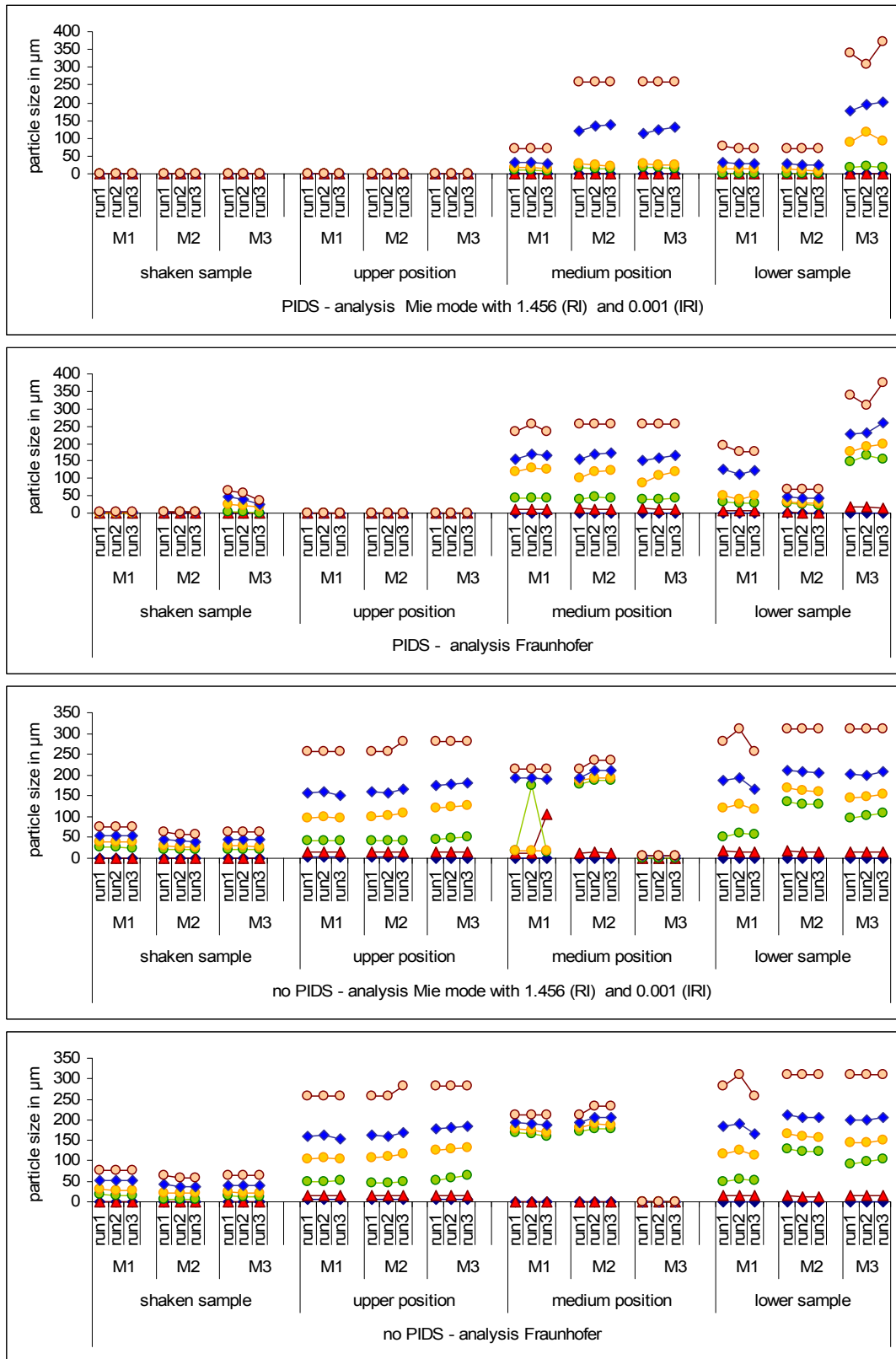
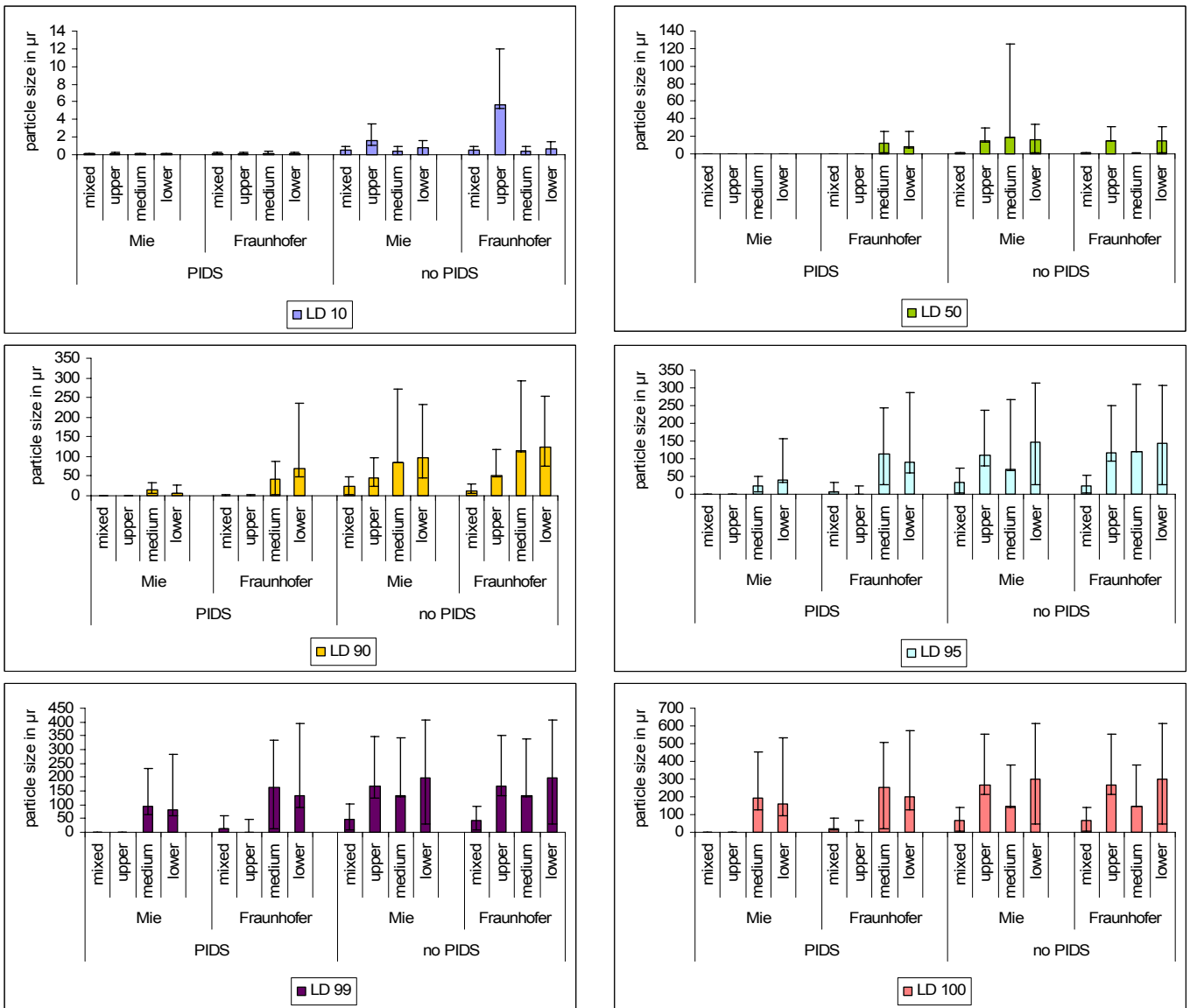


Figure 4-51: Influence on results if the sample is collected from different positions; sampling was performed from the top (upper position), middle (medium position) and the bottom of the vial (lower sample) and after shaking the vial (shaken sample)

Table 4-17: standard deviations for LD 10, LD 50, LD 90, LD 95, LD 99 and LD 100 of the measurements obtained from batch Stab. 3



The graphs in Table 4-17 show the different results obtained for the width of the distributions, the LD 10, LD 50; LD 90, LD 95, LD 99 and LD 100. Samples have been measured with PIDS and without PIDS technology and were analysed with Fraunhofer and Mie mode. As already shown in 4.7.3, measurements without using the PIDS technology enhance the probability for the detection of larger particles beside a smaller bulk population. The reason might be the fact, that the required volume of sample for analysis is about 5-10 orders of magnitude higher than the volume required for a measurement with included PIDS technology. Therefore, the amount of particles at all, as well as the amount of larger particles within the analysed sample, increases. However, the aim of this experiment was the investigation of the impact on the result due to the sampling from different positions within the sample. The position of sampling seems to be very important for the result obtained, when

the sample is not monodisperse. It was found that sampling from a shaken sample and from the upper position of the sample did not lead to the detection of larger particles, when PIDS technology was included. Sampling from a medium position and a lower position led to the detection of larger particles. When PIDS technology was not included, larger particles were detected in all sampled positions. Also here the particles detected from the shaken sample were smallest; the largest particles were detected from the lower position. The LD 50, which corresponds to the 50% volume diameter, was small in the shaken sample and in the sample drawn from the medium position, but was high in the upper and lower position. This indicates that larger particles were only collected in the upper and lower position of the sample. Sampling in the shaken sample and in the medium position could collect small and larger particles, leading to analysis of LD 50 being representative for the particle population.

4.7.4.2 Conclusion

The position of sampling is important for the result. If too less large particles exist beside a small bulk population, they might be overseen by the analysis, leading to wrong results, where it is assumed, that no larger particles exist. The most misleading analysis in this experiment was measurement when PIDS was included and sampling took place from a shaken sample or from a medium position of a non-shaken sample. This mostly corresponds to the sampling suggested by (Beckman-Coulter 1994; Müller 1996) and is therefore the most performed way of sampling. Shaking and drawing from the middle is a representative sampling. However when one wants to be highly sensitive against larger particles in stability studies one should draw samples from positions where large particles are enriched in non-shaken vials (i.e. sediment or flotation top layer zone). Of course it needs to be kept in mind that these are non-representative samples!

When PIDS was excluded the larger particles could be found. Therefore it is suggested, that a measurement of one sample should include a measurement with PIDS and one measurement without PIDS. Also it is suggested that, if the aim is the detection of the largest particles, the sampling should take place in the upper or lower position of the sample container, depending on the nature of sample (e.g. emulsions might float, whereas suspensions show sedimentation). If only a small amount of sample is available measurements without PIDS are limited, because of the high amount of sample required for one measurement. Here it is suggested to analyse the sample twice. Both measurements can include PIDS. The first measurement should be performed using a rested and non shaken sample in order to ensure the inhomogeneous probe. The sample should be collected from the upper or the lower part of the sample, depending on the nature of the sample. The second measurement analyses the

mean particle size of a representative sample. Here, the sample should be shaken and collected from the middle of the sample container. From all this it is also recommended to control the results by an independent characterisation method. Light microscopy is suggested; as it is easy and gives clearly visible results.

4.7.5 Temperature controlling

The LS 230 has no temperature control. Also other instruments on the market are not equipped with temperature control systems. In general it is assumed that this technique is not sensitive to changes in temperature. However, the refractive index is needed for correct submicron particle size analyses and is sensitive to temperature. Also it was shown, that particles can dissolve during the measurement. The saturation solubility as well as the dissolution velocity are temperature dependent. If particles dissolve, the particle size distribution would be too broad or too narrow and the particle size too small or too large (see 4.7.2.1). If NLC or SLN are analysed the increase of temperature may reach the melting point of the particles, leading to a different shape of irregular particles, which can cause a change in particle size. From this it is clear that the temperature is an important factor of laser diffractometry but completely neglected in the literature about laser diffractometry. No literature is available about changes in temperature during the measurement. In general it is assumed, that measurements are performed at room temperature. The corresponding indices of refraction are used.

4.7.5.1 Increase of temperature - influence on start temperature

The aim of the experiment was the investigation of changes in temperature during the measurement. 20 samples have been analysed using the LS 230. PIDS was included into the analysis. Each measurement included three single repetitions.

The temperature was measured:

- in the measuring medium prior adding it into the measuring cell (bulk container)
- after degasing the measuring medium (de-bubble process)
- after adding the sample (start of measurement)
- after the third run (end of measurement)

The data obtained are shown in Figure 4-52.

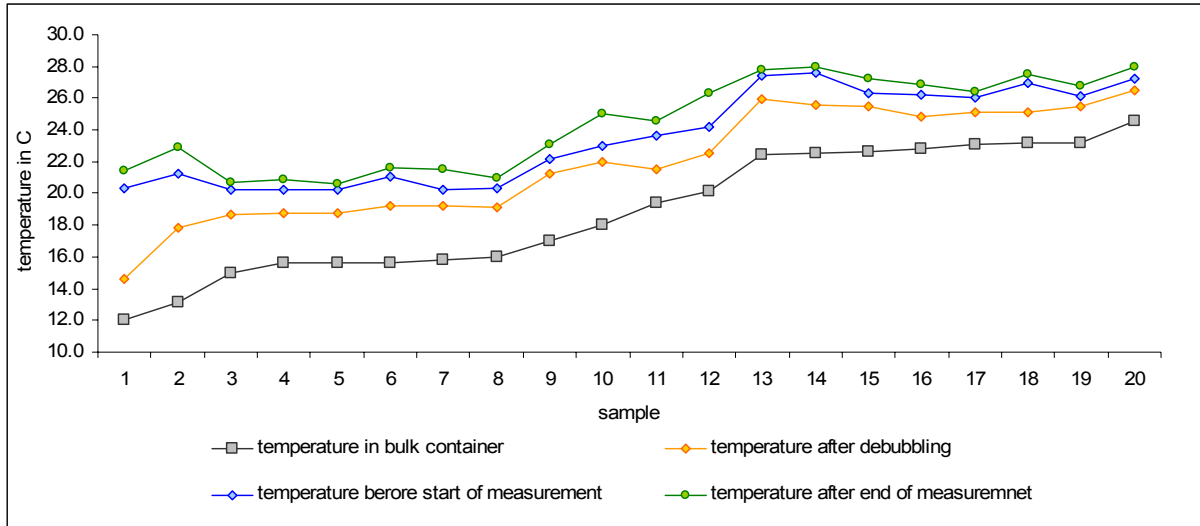


Figure 4-52: Increase in temperature during the measurement for 20 samples with different start temperatures of the measuring medium

In Table 4-18 the obtained data are listed. The table also includes the calculated increase of temperature during the measurements. The differences between the temperatures in the bulk container, prior filling and until the end of the measurement are viewed in the fifth column. The increase during the measurement itself (difference of temperatures from start of the measurement until the end) is shown in the sixth column.

Table 4-18: Increase in temperature during the measurement for 20 samples with different start temperature of the measuring medium

	temperature in bulk container	temperature after debubbling	temperature before start of measurement	temperature after end of measurement	difference of temperature before filling until end of measurement	difference of temperature from start till end of measurement
1	12.0	14.6	20.3	21.4	9.4	1.1
2	13.1	17.8	21.2	22.9	9.8	1.7
3	15.0	18.7	20.2	20.7	5.7	0.5
4	15.6	18.8	20.2	20.9	5.3	0.7
5	15.6	18.8	20.2	20.6	5.0	0.4
6	15.6	19.2	21.1	21.6	6.0	0.5
7	15.8	19.2	20.2	21.5	5.7	1.3
8	16.0	19.1	20.3	21.0	5.0	0.7
9	17.0	21.2	22.2	23.1	6.1	0.9
10	18.0	22.0	23.0	25.0	7.0	2.0
11	19.4	21.5	23.6	24.6	5.2	1.0
12	20.1	22.5	24.2	26.3	6.2	2.1
13	22.4	25.9	27.4	27.8	5.4	0.4
14	22.5	25.6	27.6	28.0	5.5	0.4
15	22.6	25.5	26.3	27.2	4.6	0.9
16	22.8	24.8	26.2	26.9	4.1	0.7
17	23.1	25.1	26.0	26.4	3.3	0.4
18	23.2	25.1	27.0	27.5	4.3	0.5
19	23.2	25.5	26.1	26.8	3.6	0.7
20	24.6	26.5	27.2	28.0	3.4	0.8

4.7.5.2 Conclusion

Temperature increased during the measurement in the LS 230. The increase was time dependent and dependent on the temperature of the measurement medium prior added to the measurement cell. The lower the start temperature, the higher is the total increase in temperature during the measurement. Measurement medium having room temperature (average 23.0°C prior adding to the sample cell, led to final temperatures of about 28 °C at the end of the measurement. When very cold measurement medium was used the temperature did not exceed temperatures above 22°C. Therefore samples sensitive to increases in temperature can also be analysed, by using cooled water.

4.8 Summary

This study showed that the results obtained from laser diffractometry using the LS 230 are highly variable, depending on the many factors. The most influencing factor was found to be the choice of the optical parameters. The choice of the optical parameters influences the analysed particle size diameters and the shape of the size distribution as well as the width of the distribution. Fraunhofer approximation was not sufficient for the characterisation of small particles; also incorrect values for refractive indices can yield results being far away from the reality. Therefore only the correct input of optical parameters can yield reliable results.

LD results can be analysed as volumetric, surface specific or numeric distribution. Volumetric distribution is the most common method of analysis, however if small particles exist beside a larger population this mode might fail to detect the small particles. In those cases numeric analysis can be additionally applied, as here the impact of smaller particles is increased and vice versa the impact of larger particles is decreased. Hence, larger particles are not detected in this mode. In conclusion a full characterisation in such cases should include both – numeric and volumetric analysis.

The PIDS technology for the enhanced detection of small particles is used in combination with the basic LD measurement (diffraction). It was found that the purpose of enhanced resolution of even trimodal dispersions in the submicron range by using only Fraunhofer approximation could not be approached for the polydisperse latex dispersions analysed here. The method failed either in detecting the different size populations or in correctly calculating the correct volume distributions. From this it is concluded, that also nanosuspensions and NLC having a polydisperse but small sized particle distribution can not be perfectly analysed. Therefore results remain an (useful) approximation. Of course this is valid for polydisperse nanosised dispersions in general. Nevertheless even though the resolution was not as sensitive as expected and the volume distribution failed to be correct, if correct optical parameters were selected the results still gave information being useful to distinguish monodisperse systems from polydisperse systems, which is the most important fact, when analysing nanosuspensions or NLCs.

Also other influencing parameters investigated were identified to be crucial for correct measurements. Here the most important finding was that including PIDS into the LD measurement can disable the detection of large particles within a sample which mainly consists of small particles. The detection is even no not reliable when the sample is collected from places of an enrichment of larger particles (e.g. sediment of vial) if PIDS is included into the measurement. Only if PIDS is not included into the measurement, large particles are

reliably detected. The disadvantage of the diffraction method is the smaller measuring range from only 400nm - 2000 μ m, particles from 40nm upwards to 2000 μ m can be only analysed when PIDS is included. Also the amount of sample needed is extremely higher for measurements without PIDS, but can not be avoided in this case.

The dissolution behaviour of nanosuspensions was also subject to this study. It was found that dissolution has an extreme impact on the particle size and size distribution analysed. Depending on the time of the measurement, on the dissolution velocity of the drug and on the temperature, the size distribution can be too broad or too narrow. Using numeric analysis in those cases will lead to absolutely false results. Dissolution can only be avoided by using nanoparticle saturated measuring medium. Dissolution effects of nanosuspensions have a much smaller impact in the result when PIDS is not included. Due to the large amount of added sample and the lack for the detection of particles smaller 400nm, the dissolution effects can be overwhelmed.

Moreover the temperature of the sample was studied during the measurement. Interestingly it was found that the temperature increases steadily over the time of the measurement. The main increase occurs before the measurement itself, during the set up of the measurement (e.g. alignment, debubbling, input of sample name and details). During the measurement itself the temperature is only slightly increasing further. However the averaged temperature for a standard measurement (60s per measurement and 3 repetitions) was found to be 28.3 °C +/- 0.5°C. The impact on results rises as other influencing parameters are temperature dependent. The saturation solubility is temperature dependent. Therefore nanosuspensions can dissolve under those conditions even if the measuring medium was saturated. This is because saturation is normally performed at room temperature. In contrast cyclosporine is inversingly soluble to the temperature. Here re-crystallisation of nanosuspensions was observed due to the increase in temperature. The problem can be overcome if cold water is used as measuring medium instead of water having room temperature. To be correct, measurements should therefore be performed in a standardised way, where the time of the measurement and the corresponding temperature are controlled and documented.

In the past measurements were analysed mostly without the knowledge of the optical parameters. Nanosuspensions were analysed using Fraunhofer approximation. For NLCs the optical parameters 1.456 and 0.01 were used after (Müller 1996). As suggested from the manufacturer PIDS was always included into the measurements. Temperature control was never performed and saturation of the measuring medium was randomly performed only in those cases where the nanosuspension dissolved obviously. Mostly no saturation was

performed, the amount of sample added to the instrument not reported and possible changes in obscuration were not observed (or even ignored) and not documented. In the following this way of performing LD measurements is referred to the “conventional method” of characterisation. Errors of the conventional method were identified within this thesis and are summarised in 4.8.1.

4.8.1 Errors of the conventional method

The main object to characterisation of submicron particles by laser diffractometry – apart from information about the bulk population - is the detection of possible large particles. Other techniques, mainly photon correlation spectroscopy (PCS) are more appropriate to gain information about the main particle size of submicron particles, because PCS does not require any knowledge about scattering properties such as refractive indices. In addition PCS is highly sensitive to detect even small changes in the mean size of the bulk population (S.D. 1% only!), being the reason why it is typically combined with LD for nanoparticulate systems.

The occurrence of large particles indicates instability of the system (or poor preparation). Therefore early detection of those particles is the main interest if long time stability studies are performed. Another early indicator of instability is an inhomogeneous system a priori after production, where more than one population is present. Thus, an accurate size characterisation should ensure the correct resolution of particle size populations. Also an increase of the main particle size over time indicates changes of the system and therefore instability and must be correctly analysed by a method.

Using the “conventional method” to perform LD measurements none of these requirements could be ensured, due to the following reasons:

- incorrect particle sizes and size distributions are caused by using only Fraunhofer approximation or in case of Mie, using guessed standard optical values for all systems characterised
- the detection of larger particles mostly fails due to the inclusion of PIDS into the analysis of the measurements
- microscopic analysis to control the absence of larger particles in parallel to each measurement is usually not performed due to saving time
- non observed dissolution effects lead to smaller or larger particle sizes and broader or smaller size distributions.
- non standardised measurements lead to results, varying depending on the daily performance, making an objective comparison and interpretation of results, e.g. increases or decreases in particle size impossible

4.8.2 Optimisation of LD-measurements using the LS 230

From the results of this thesis the following modifications are suggested to improve the results obtained by laser diffractometry when characterising submicron particles, e.g. nanosuspensions and lipid nanoparticles (SLN, NLC).

4.8.2.1 General suggestions

Fraunhofer approximation should only be applied for particles at least six times larger than the wavelength of the laser beam. For all systems smaller Mie theory should be applied. For that the real refractive index and the imaginary refractive index must be known for the particles, also for the measuring medium the real refractive index is required. The input of guessed values surely leads to incorrect results.

Using the LS 230 samples should be analysed performing two measurements, first with included PIDS, second without PIDS. The measurement with PIDS can gain information about the main particle size and shape of size distribution. Measurements without PIDS ensure the detection or the absence of larger particles.

Two measurements for only one sample are time consuming. Therefore, here a more convenient method is suggested. In practice the main time consuming procedure is the set up of the instrument, i.e. rinsing, filling, de-bubbling, alignment and background measurements which takes approximately 5 min in total. The measurement itself takes 3 minutes if PIDS is included. Measurements without PIDS only take 1.5 minutes. In this work a method was established where both measurements can be performed subsequently. The time consuming steps from filling to background measurement can be saved, if one directly enters the start up menu (run > run cycle) after the last run of the measurement with PIDS is finished, without flashing the sample cell. This is possible as the same sample will be analysed and the concentration of sample needed for measurement without PIDS is much higher. If the start up window is open one continues by pressing the bottom new sample and deleting the signs (crosses) at background measuring and PIDS, the buttons “measuring sample load” and “perform 3 runs” must be ticked. Under run preferences the time of measurement can be changed from 60s to 30s and under “sample info” the sample name can be changed. It is suggested to rename the sample by only adding nP (no PIDS) behind the first sample name. This ensures that the two corresponding measurements with and without PIDS are automatically arranged underneath each other in the database of the PC. Databases manage the data by alphabetical order. If the measurement without PIDS is renamed, the PC rearranges the saved data in an alphabetical order, leading to disruption of the corresponding measurements. Re-shuffling of the corresponding measurements would waste a lot of time

and should be avoided. After turning back to the main start up window the measurement can be started by pressing the button “start” measurement. The measurement than can be continued as usual. The sample load for measurements without PIDS should be 7-10% (Beckman-Coulter 1994).

Attention must be drawn if the sample is sensitive to changes in temperature, as the temperature increases over the time of the measurement. In those cases the convenient measurement method one after the other without flashing is not advisable.

All samples should be analysed in parallel using light microscopy. The findings here, clearly gave evidence that if large crystals are present within many small particles, that they can also be overseen by this characterisation method if polarised light is not applied. Therefore also this method must be performed with caution. Best results were found if small magnifications (e.g. 160x) with and without polarised light were used to gain an overview of the systems. At least three different samples should be drawn for each system. If no crystals are detected within the first sample taken, further samples should be collected from places where higher concentrations of large particles are expected (e.g. bottom of the vial). This procedure enables the detection of large particles at a very early stage. Large magnifications (e.g. 100x) are not useful for the detection of larger crystals. They can be used to gain detailed information of single particle sizes.

For microscopic analysis it is highly important **not** to dilute the samples. Non-diluted samples increase the probability of finding even a few large particles, as it is not the aim to see the nanoparticles with a high resolution, but only to make a search for the possible large particles (i.e. $>1\mu\text{m}$). This approach of analysing non-diluted dispersions was already performed about 20 years ago by Müller and Heinemann (Muller and Heinemann 1991).

4.8.2.2 Size characterisation of nanosuspensions

Size characterisation of nanosuspensions in general should be performed as described above. Additionally dissolution must be avoided in order to avoid changes in particles sizes. As the progress of dissolution is time and temperature dependent, the time and temperature of each measurement should be kept constant. The varying parameters of the measurement are the time were sample is added to the sample cell of the instrument and the input of the sample details. The input of the sample details in the conventional method is performed after the sample was added, but can be done within the set up (in the start up window), which is suggested. The time needed for the addition of the sample can be validated.

The measuring medium needs to be saturated, preferentially with the nanosuspension itself. The saturation of the measuring medium should be performed at temperatures around 28°C as

this is the temperature, where the measurement is performed. If the medium is saturated at room temperature, further increase in temperature during the measurement can increase the saturation solubility, leading to dissolution of the nanosuspension. However, changes of temperature of such a highly saturated medium can also lead to re-crystallisation effects. Re-crystallisation can occur if the saturation solubility decreases with increasing temperature or the supersaturated systems get a “crystallisation initiation” turning to a “normally” saturated system under microcrystal formation. Therefore the obscuration values of the actual measurement should be controlled before and after each measurement. Dissolution of the sample is indicated by a rapid decrease in PIDS obscuration. Re-crystallisation effects are indicated by an increase in obscuration of the LD cell. Changes of the system over time can also be detected by only comparing the obscuration values. If the amount of sample added to the instrument is kept constant for each measurement an increase in LD obscuration and a decrease in PIDS obscuration clearly indicate particle growth.

However, the suggested procedure is extremely time intensive. The question arises if the information gained from PIDS measurements is crucial for the characterisation of nanosuspensions and if the effort needed for those measurements is really worth it. Changes of particle size for small particles can be detected by using photon correlation spectroscopy. Microparticles $>3\mu\text{m}$ cannot be detected by PCS because they are outside the measuring range, even particles $>1\mu\text{m}$ are difficult to resolve because it requires sophisticated Fourier transformation of the correlation function. The main subject of LD measurements remains therefore to be reliable in the detection of larger particles, which is only ensured if PIDS is not included into the measurement. Therefore it is concluded, that LD measurements for nanosuspensions are most effective if only measurements without PIDS are performed. In those measurements the impact of dissolution effects is much smaller. This is because the measuring range is only from $400\text{nm} - 2000\mu\text{m}$, where the small particles which are subject to those effects are not analysed. Also the much higher volume of sample required for measurements without PIDS reduces the impact of some dissolved particles to the all-over result.

However dissolution also occurs within measurements without PIDS. Therefore also here the measuring medium needs to be saturated. The increase in temperature of the measuring medium during the measurement is also a subject for consideration, as re-crystallisation or further dissolution may occur. From the experience of this work the following procedure was found to be the most effective solution:

The measurement is performed as described in the general part, also here the two measurements with and without PIDS are conducted. In contrast to the general part, here PIDS measurements are not performed to gain information about the particle size but to observe the saturation progress of the measuring medium. The nanosuspension is added to the instrument until a sufficient high PIDS obscuration is reached. Over time the obscuration decreases, as the suspension dissolves. New sample is added than until the obscuration keeps constant. One measurement (only one run) can then be conducted. Results from those samples analysed with PIDS does not yield correct results as the ratio of small and large particles does not fully correspond the original suspension. Therefore they should not be used for further assessments. The measurement is only performed to yield access to the start menu from which now the measurement without PIDS can be started. The results of such measurements without PIDS lead to correct results, because the amount of freshly added sample is about ten times higher than the amount which remained from the saturation process. If the sample is sensitive to changes in temperature this measurement method one after the other without flashing is not advisable. For such systems it is advisable to use saturated measuring medium instead of unsaturated water. If fully saturated medium is used the saturation procedure does not need to be performed. Therefore the time of the measurement is decreased and the all over increase in temperature during the measurement as well. Further reduction of the temperature can be gained if the saturated measuring medium is cooled prior the measurement.

All parameters explained here should be controlled and documented.