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Size analysis of submicron particles by laser diffractometry—90% of the published measurements are false

Cornelia M. Keck *, Rainer H. Müller

Department of Pharmaceutical Technology, Biopharmaceuticals & NutriCosmetics, Free University of Berlin, Kelchstrasse 31, 12161 Berlin, Germany

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Abstract

The first part of this paper gives an easy and understandable introduction into modern laser diffractometry (LD). It explains why modern laser diffractometry is not only the simple detection of a diffraction pattern but a combination of two independent technologies, which is done to extend the measuring range down to several nanometers and incorrectly named LD measurement. In the second part the impact of the optical parameters on the result analysed was investigated. The results show, that changes in optical parameters change the particle size and the size distribution tremendously. For a trimodal mixture of latex particles, having a mean particle size of 845 nm, mean particle sizes ranging from 284 nm to up to 1.005 μm were obtained due to the variation of the optical parameters. The obtained particle size distributions varied from monomodal, bimodal over trimodal to even tetramodal distributions. The results prove that the analysis of submicron particles is meaningless, if incorrect optical models are applied. Another hazard was found to be the use of the additional techniques for the extension to the submicron measuring range. Combining this technology with laser diffraction can fail to detect larger particles besides a small sized main population. This can be overcome by analyzing the samples also without the additional technology, i.e. using laser diffraction only. From the results it is concluded that laser diffractometry for submicron particles is only useful if correct optical parameters are applied and if the presence of larger particles is investigated without the enhanced submicron measuring range.

Keywords: Laser diffractometry; Fraunhofer; Mie; Refractive index; Nanoparticles; PIDS

1. Introduction

The determination of the particle size and the size distribution is frequently performed in many laboratories by using laser diffractometry (LD), which is also known as small angle static light scattering. This technique is very popular, because of its easy use and its accurate reproducibility (<1%) (Malvern, 2007; Beckman-Coulter, 2007a; Sympatec, 2007b). However, the biggest advantage of this method is its especially outstanding broad measuring range. Whereas older instruments could analyse particles from 400 nm up to several mm (Beckman-Coulter, 2007b; Malvern, 1997), new instruments also analyse particles up to several mm but possess an extended measuring range for submicron particles, enabling the analysis of very small nanoparticles (e.g. 20 nm) (Malvern, 2007; Horiba-Instruments, 2007). The extension of the measurement range was just in time with the increasing interest of nanoparticles in the pharmaceutical industry. With that LD measurements can be applied for microparticles, macroparticles and nanoparticles and mixtures of those, nearly making this technique an all-purpose technique.

Nevertheless, nothing is perfect. Therefore, also this technology has limitations and problems, which are not easy to overcome, but often overseen, underestimated or simply unknown to the user. One example is the use of the optical parameters for the analysis of submicron particles. By theory for a correct analysis result the use of optical parameters is necessary if the particle size is smaller than six times the wavelength applied for analysis (Mie, 1908; Römpp, 1995). Thus the FDA established guidelines in 1999 (ISO 13320, 1999), which suggest that for a correct analysis of submicron particles optical parameters should be applied. Optical parameters are compound specific and unfortunately they are not known for most of the solid pharmaceutical compounds (Römpp, 1995; Rawle, 2004) and/or hard to access (Rawle, 2003, 2004, 2006). Therefore, it is

* Corresponding author. Tel.: +49 30 83850631; fax: +49 30 83850631.
E-mail address: ck@ckc-berlin.de (C.M. Keck).

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common habit to use the Fraunhofer approximation, e.g. seen in (Beckman-Coulter, 1994; Onofri et al., 2004; Dailey et al., 2003; Leterme et al., 2005; Wabel, 1998), where no optical parameters are required or standard optical parameters, already stored in the software of the instrument, e.g. in (Akkar and Müller, 2003; Jores et al., 2004; Moschwitzer and Müller, 2006; Worle et al., 2006) are used. Also manufacturers force this way of analysis, because it is also common sense to expect the influences of the optical parameters on the analysed result to be little or neglectable (Beckman-Coulter, 1994; Sympatec, 2007a; Kippax, 2006). By screening the literature it becomes obvious that in most of the cases no information about the analysis mode is given at all (e.g. in (Saupe et al., 2006; Turner et al., 2004; Rosca et al., 2004; Uner et al., 2005; Rabinow et al., 2007)), which proves that no attention is put on this fact until now.

In addition laser diffractometry is also in focus of criticism, because the comparability of results between different laboratories or even between different experiments often fails due to unknown reasons. There is at least one publication which reports the un-usefulness of the method at all (Driscoll et al., 2001). The authors state, that the reliability of the method is not appropriate for analyzing multimodal samples (e.g. large particles besides a small sized main population) and therefore suggest using different methods rather than laser diffractometry. Nevertheless, laser diffractometry is a frequently applied technique for the size characterization of submicron particles. Even though it is mostly used for a simple particle size characterization; its intention should be to investigate if the small sized sample also contains larger particles. This is because there are other more appropriate methods to characterize small particles alone (e.g. dynamic light scattering technologies (Müller and Schuhmann, 1996). However, on the other hand side, there are not many methods for the detection of larger particles besides a small sized main population, because the upper detection limit for most of the methods is only a few μm (Müller and Schuhmann, 1996; Rawle, 1993). Hence LD is the method of choice for the detection of larger particles within a submicron system. Larger particles in a submicron system can occur due to stability problems (e.g. agglomeration of particles or coalescence in emulsions). If such changes of a system are overlooked this would be very crucial, e.g. an emulsion for intravenous administration containing small droplets with a size of 250 nm as main fraction, where laser diffractometry is used to monitor the physical stability of this emulsion. Clearly the user is interested to ensure that no larger droplets, which could cause capillary blockade after administration, occurred over the time of storage. Obviously, in this case it is from secondary interest how exactly, the particle size of the small particles is distributed (e.g. some smaller particles of 100 nm and some larger of 500 nm). Taken this example, it must be emphasized again, the primary intent for most of the LD measurements analyzing submicron particles is to clarify whether or not the system contains any larger particles.

2. Aim of work

From our experience it is hard to find an easy introduction to laser diffractometry, covering the basics of the technology. Therefore, the first intention of this paper is to give a very simple and understandable introduction into the modern LD technology. The second part of the paper investigates the impact of the optical parameters on the analysis result obtained, to clarify whether the use of optical parameters is truly essential (as suggested by the ISO 13320) or not (common habit). To investigate the usefulness of the method, which was doubted by Driscoll et al. (Driscoll et al., 2001) in the third part the reliability for the detection of larger particles besides a small sized main particle population was investigated.

3. Materials and methods

3.1. Materials

For the measurements the laser diffractometer LS 230 from Beckman-Coulter (Krefeld, Germany) was used. The analysis and simulations of the optical parameters were performed by using the Beckman-Coulter software, version 3.19.

Standardized latex particles (102 nm, 204 nm, 404 nm, 845 nm and 2.875 μm) were obtained from Postnova Analytics (Landsberg, Germany). The nanostructured lipid carrier (NLC) dispersion was produced via high-pressure homogenization stored at 4 °C and analysed 28 days after production. It was loaded with tretinoin (0.30 wt%) and contained stearyl alcohol (17.73 wt%) and Miglyol 812 (1.97 wt%) as lipid matrix, Tween 80 (2.00 wt%) as emulsifier and water (78.00 wt%).

3.2. Methods

All LD measurements were performed as suggested by the manufacturer (Beckman-Coulter, 1994). Measurements were performed with enhanced submicron measuring range (PIDS) and without. The run lengths for the measurements with included polarization intensity differential scattering technology (PIDS) was 60 s, for measurements without PIDS (i.e. analysis with laser diffraction only) was 30 s. The obscuration was adjusted to 40–50% for the measurements with included PIDS and to 7–12% for the measurements without PIDS. All measurements were performed in triplicate and the results were analysed as volumetric distribution (Rawle, 1993). Additionally light microscopy was performed with and without polarized light using a Leitz-Orthoplan microscope (Wetzlar, Germany) and appropriate magnifications, e.g. 160× for the detection of possible larger particles and 1000× for the evaluation of smaller particles.

4. Theoretical background of laser diffractometry

Laser diffractometry uses the fact that the pattern of diffracted light is dependent on the particle size of the sample to be analysed (Müller and Schuhmann, 1996). Though, the principle of LD is the detection of the created diffraction pattern derived from an illuminated particle (Horiba-Instruments, 2004). This phenomenon is very complex, but might be explained (more or less scientifically) using a simple stone, which drops vertically into water as an example. When a stone hits the water surface

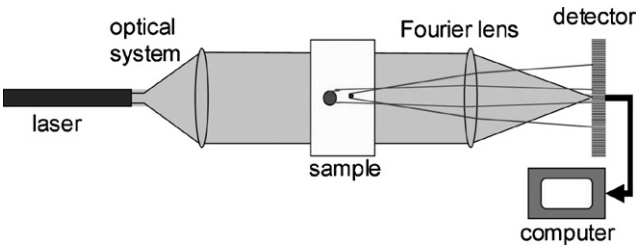

Fig. 1. Basic setup of a laser diffractometer modified after (Müller and Schuhmann, 1996), from left to right: light source (laser) to illuminate the particles, optical system to widen the laser beam, sample cell, Fourier lens to collect the diffracted light, ring detector, PC (software) to calculate the particle size.

waves, which look like rings of different sizes around it, will occur. The ring being closest to the stone will be very intense, whereas the height of the outer rings decreases with an increasing distance from the stone.

Depending on the size of the stone, the pattern of the waves will be different. A large stone will create higher waves, when compared to a very small stone. Additionally the distance between the single lobes is lower in case of the larger stone. In principle, this is exactly what happens, if light hits a particle. In fact LD measures the intensities and the distance of the rings, which are created upon illumination of a particle. Therefore, the basic set-up of laser diffractometers is the same. It consists of a light source (laser) to illuminate the particles, an optical system to widen the light beam, the sample cell, a Fourier lens to collect the diffracted light and a ring detector system to detect the created pattern. Finally this information is used to calculate the particle size via the software. Fig. 1 shows the basic set-up of a laser diffractometer (modified after (Müller and Schuhmann, 1996)). For analysis the particles need to be dispersed, where the dispersion medium can be either a transparent liquid (e.g. water, alcohol, etc.) or a gas medium (e.g. air). Thus it is possible to analyse not only liquid dispersions (e.g. suspensions, emulsions) but also aerosols.

If this would be all, laser diffractometry would be easy. Unfortunately, this is not the case, because when light hits a particle it is not only diffracted, but also:

1. *reflected* (abrupt change in the direction of a wave front at an interface between two dissimilar media, e.g. wave front returns into the medium from which it originated),
2. *refracted* (bending of light due to a change in its speed),
3. *absorbed* (transfer of energy carried by light waves to particles) and
4. *re-radiated* (as heat or light).

Fig. 2 shows the different phenomena in a simplified or way.

Hence, if light strikes a particle, it is not only a diffraction pattern, which is created, but a more complex pattern, which spreads light in every direction (Stratton, 1941; Mie, 1908). All phenomena taken together are named scattering. Therefore, the pattern created is named the scattering pattern of a particle. The shape of a scattering pattern depends on the size of the particle, and to be precise on the ratio of the particle size to the incident wavelength. Hence, the shape of a scattering pattern will not only change due to a change in the particle size with a constant wavelength but it will also change if the particle size is kept constant and only the incident wavelength is changed. Depending on the ratio of particle size to wavelength one distinguishes three major shapes of scattering patterns. Which are the:

1. Fraunhofer scattering
2. Mie scattering and
3. Rayleigh scattering

A particle being much larger, than the incident wavelength will show a Fraunhofer scattering pattern and particles with particle sizes much smaller (i.e. 10×) than the incident wavelength show the so-called Rayleigh scattering. All particles in the intermediate region show Mie scattering (Mie, 1908; Stratton, 1941; van de Hulst, 1981). In Fig. 3 the shape of the three different scattering envelops explained above are shown.

Fraunhofer scattering is characterized by a strong forward scattering and a relatively weak backward scattering. With Mie

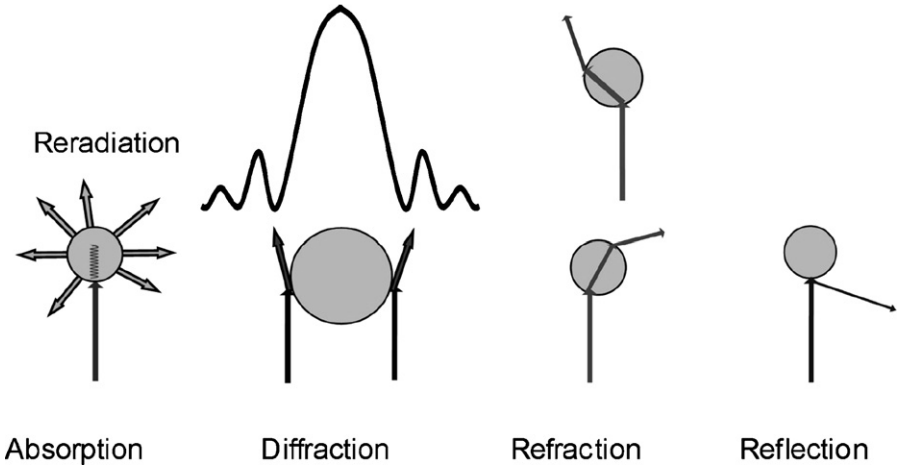

Fig. 2. Overview of scattering phenomena which occur if a particle is illuminated with light: from left to right: absorption, diffraction, refraction and reflection.

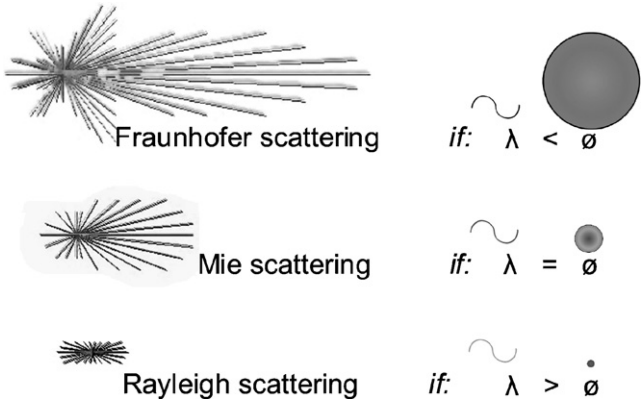

Fig. 3. Envelops of scattering as function of ratio particle size to wavelength: upper: Fraunhofer scattering (particles much larger than wavelength), middle: Mie scattering (intermediate region, e.g. particles about the size of the wavelength) and lower: Rayleigh-scattering (particles much smaller than wavelength) (λ = wavelength).

scattering the ratio of forward scattered light to backward scattered light is much smaller, when compared to Fraunhofer scattering. With Rayleigh scattering the intensity of forward scattered light is about the same as the backward scattered light intensity.

Thus, the pattern which is created by the particle upon illumination and collected on the detector system of a laser diffractometer is not only a diffraction pattern, but a complex scattering pattern. Unfortunately, there is no detector that can differentiate between diffracted light only and the other phenomena. Therefore, also all other light scattering phenomena need to be taken into account for a correct LD analysis result.

How is this possible? The solution is the application of the so-called Mie formula for analysis, which was developed by Mie (1908). The formula describes all phenomena, which occur if light hits a spherical particle. It is very complex, as it contains Bessel functions, which are probably not in everyone's repertoire. A simplified version of the Mie formula is shown in Fig. 4.

Nevertheless, by ignoring the mathematical problems, the problem regarding LD might be explained by simplifying the formula extremely. By extracting everything complicated of the formula only three influencing parameters remain. They are the:

1. *particle radius* (*A*), which is the parameter of interest and to be measured
2. *angle of scatter* (*W*) which is measured on the detector and the
3. *optical parameters* (*m*) = imaginary and real refractive index (expressed as complex refractive index).

In conclusion the particle radius can be calculated by using the Mie formula if the angle of scatter and the optical parameters are known. From this it is clear, that if the optical parameters are not known, no correct result can be expected from theory!

Basically, the Mie formula consists out of three main parts (Horiba-Instruments, 2004) see Fig. 4. The optical parameters are only incorporated in one of the three parts (Rayleigh-term) and do not occur in the other two parts. Hence, if there would be a case where this term would become so small to be cancelled out, no optical parameters would be necessary for analysis and indeed—this is the case for very large particle diameters, where the angle of scatter becomes very small. In such cases mathematically this term cancels out (Horiba-Instruments, 2004). As a consequence no optical parameters are required for the analysis of large particles. In practice this is known as Fraunhofer analysis or Fraunhofer approximation. Fraunhofer scattering occurs if a particle is at least 5–6 times larger than the incident wavelength. Therefore, in practice Fraunhofer analysis can only be used if the particles of the sample are five to sixfold larger than the wavelength of the instrument used for analysis (Müller and Schuhmann, 1996). All particles being smaller must be analysed using the complete Mie-formula, where the optical parameters are required. In practice the wavelengths of the instruments range from 633 nm to 800 nm and it is 750 nm for the LS 230. Thus, with the LS 230 only particles larger than 4.5 μm can be analysed using Fraunhofer approximation, all particles being smaller must be analysed using Mie theory. For the instruments using 633 nm (e.g. Mastersizer 2000 (Malvern), LA-920 (Horiba), Helos BF/Vario (Sympatec)) the critical size is smaller being only 3.8 μm. However, if coloured pigments are analysed Mie theory should be used for all particles being not larger than 40 times the wavelength (ISO 13320, 1999).

$$I(w) = E\{k^2 A^4 [J1]^2 W^{-1} + [K_1 W]^1 + [K_2 W]^3 + [K_3 W]^5 + k^4 A^6 (m-1)^2 W^6 / 8\pi^2\}$$

Fraunhofer Term — Rayleigh Term

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

Fig. 4. Simplified version of the Mie formula modified after (Horiba-Instruments, 2004).

$$\frac{\sin\theta_1}{\sin\theta_2} = \frac{v_1}{v_2} = \frac{n_2}{n_1}$$

or

$$n_1 * \sin\theta_1 = n_2 * \sin\theta_2$$

Fig. 5. Snell's Law to calculate the angle of refraction: $\sin\theta 1$ = angle of incidence, $\sin\theta 2$ = angle of refraction, v_1 = velocity of light in first medium, v_2 = velocity of light in second medium, n_1 = real refractive index of first medium, n_2 = real refractive index of second medium.

The optical parameters are expressed in two terms—the real refractive index n, which is mostly named refractive index (RI) and the imaginary refractive index n_i (IRI). Both indices are compound specific – often not known or considered – and change with changes in temperature and wavelength! The RI represents the ratio of the light velocity c in a reference medium (mostly vacuum) to the velocity v in the medium itself (i.e. in the particle). In the LD measurement the reference medium is the dispersion medium, e.g. water or air. The change in light velocity due to the change of the traveling medium also causes a change in the direction of light, which can be seen as kink in the light beam and is known as angle of refraction. The angle of refraction can be calculated by using Snell's Law (Römpp, 1995; Dijksterhuis, 2004), see Fig. 5.

The imaginary index is a measure for the strength of absorption loss and also known as extinction coefficient k (Rawle, 2003, 2004; Haas, 2002; Römpp, 1995; Wikipedia-Contributors, 2007). For the LD analysis of submicron particles both indices are important. The RI gives information, where the light intensity which did not arrive due to diffraction but from refraction can be expected on the ring detector of the instrument. Hence an incorrect RI would lead to incorrect information for the analysis of the result. The same is true for the IRI, from this index the software calculates how much light was absorbed by the particles of the sample; also here an incorrect input would lead to an incorrect analysis by theory. Therefore, using Mie theory for LD analysis requires the input of both, the real refractive index and the imaginary index. By doing this is the user establishes a so-called optical model, which can be saved under a name, chosen by the user itself. As mentioned above, the problem is that for many compounds no optical parameters are known, therefore, it was often observed, that users just use previously established optical models for there compounds to be analysed. Or even worse, just use any optical model. However, in most of the cases the users use the two standard values for RI and IRI being given by the software of the instrument.

However, there are more difficulties, which need to be considered, when analyzing samples by laser diffractometry. As seen in Fig. 3; the intensity of forward scattered light decreases, with a decrease in particle size. As a consequence the signal collected on the detector of the instrument becomes less, the smaller the particles are. Thus, every instrument has a detection limit for small particles. Depending on the wavelength of the light source the lower size limit of detection is about 400 nm (laser wavelength 750 nm, e.g. LS 230). Nevertheless, nowadays laser diffractometers can analyse particle sizes down to 20 nm (Horiba-Instruments, 2007; Malvern, 2007). As described, this is far below the detection limit of a laser diffractometer! Therefore, to extent the measuring range down to several nanometers, additional techniques to be combined with LD are required. Hence, every laser diffractometer with such a broad measuring range uses an additional technique different to pure laser diffractometry. Of course, due to intellectual property reasons, the additional techniques used are different for each manufacturer. However, all base on the same principle, which is to gain more detailed information about the shape of the scattering pattern which is created upon illumination of a particle. As explained above, particles scatter light in every direction (see Fig. 3). Therefore, it is possible to gain more information about the shape of a scattering pattern by detecting light intensities not only in forward direction, but also at other directions (e.g. backward scattering, sideward scattering). By comparing the detected intensities conclusions can be drawn about the shape of the scattering pattern. For example, nearly adequate forward scattered and backward scattered intensities lead to the conclusion, the particle generates Rayleigh scattering, whereas a very intense forward scattering accompanied with a weak backward scattering would lead to the conclusion that the particle possesses Fraunhofer scattering characteristics (Fig. 3). Nevertheless, more information is needed for a good analysis result. Therefore, most of the manufacturers use two ore more different wavelengths, to obtain different scattering patterns from different wavelengths. The trick here is the fact, that the shape of the scattering pattern changes, if the wavelength changes. Thus, a sample's scattering pattern is obtained at different wavelengths and the shapes of the different patterns obtained are compared between each other. From the differences of the patterns conclusions about the size of the sample can be drawn. For example, the LS 230, which uses the so-called PIDS technology to extend the measuring range (Bott and Hart, 1990). PIDS measures the scattered light intensities of a sample at three different wavelengths (450 nm, 600 nm and 900 nm), as in principle described above. The scattered light intensities are detected at six different angles (60°, 75°, 90°, 105°, 120° and 146°), enabling an almost complete detection of the shape of the scattering patterns. To make things a bit more complicated, in reality the shape of the scattering pattern is not only measured at three different wavelengths but also measured twice with each wavelength. First with vertically polarized light and secondly with horizontally polarized light. This procedure is necessary as the scattering pattern also depends on the polarization of the incident light. Thus PIDS analyses the differences between the horizontally and the vertically radiated light for each wavelength, to gain even more information about the particle size distribution. The three different wavelengths are created by a filter wheel, which is placed between the light source and the sample. The wheel has six pinholes, which is two for each wavelength and one for each polarization. The setup of PIDS is shown in Fig. 6.

The principle of the particle size calculation is as follows; assuming a particle having a size of 450 nm. This particle would show Mie scattering at a wavelength of 450 nm, because the size is as big as the wavelengths itself. If the same particle is illuminated with 600 nm it is smaller than the incident wavelength,

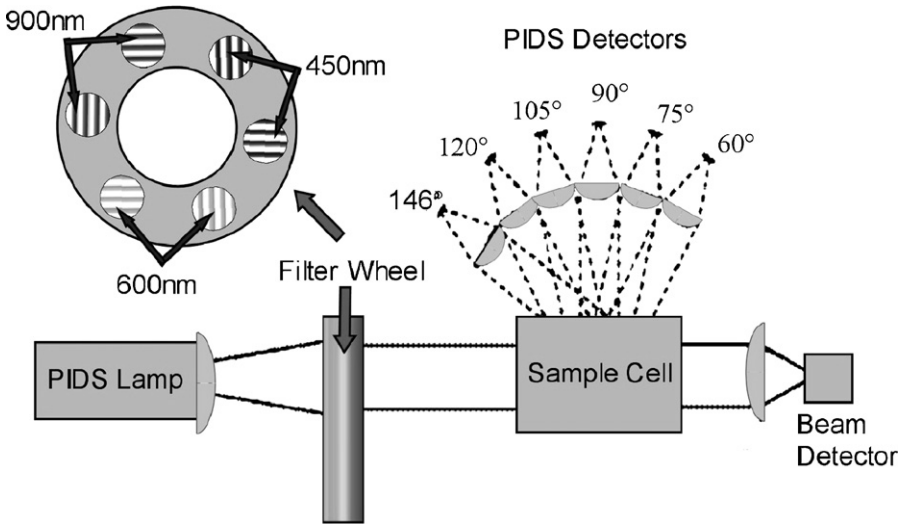

Fig. 6. Schematic setup of PIDS after (Bott and Hart, 1990). From left to right: light source (tungsten lamp), filter wheel for the generation of the different light sources (450 nm, 600 nm and 900 nm), sample cell and 6 PIDS detectors at 60°, 75°, 90°, 105°, 120° and 146° (placed at 90° to the sample cell).

thus the forward scattered intensity decreases (see Fig. 3). At 900 nm the intensity of forward scattered light is even less than it is with 600 nm, as the size of the particle is now much smaller than the wavelength applied. In contrast to that behave scattering patterns of larger particles. Imagine a large particle with a size say 50 μm. If the particle is illuminated with a wavelength of 450 nm it will show Fraunhofer scattering, however, it will also show Fraunhofer scattering at 600 nm and 900 nm, because the size is much larger for all the wavelengths applied. Hence, patterns of large particles are not much influenced by changes in wavelengths. Therefore, the greater the variation of the detected intensities between the different wavelengths is, the smaller are the particles within the sample analysed. The upper side of Fig. 7 shows the size graphs of two LD measurements. One sample corresponds to a coarse drug powder (cyclosporine powder) with a mean particle size of about 40 μm (left). The second measurement shows the particle size distribution of very small latex particles with a mean size of 155 nm (right). On the lower part of the figure the corresponding PIDS data are shown. Here, three main column groups are shown. The column groups from left

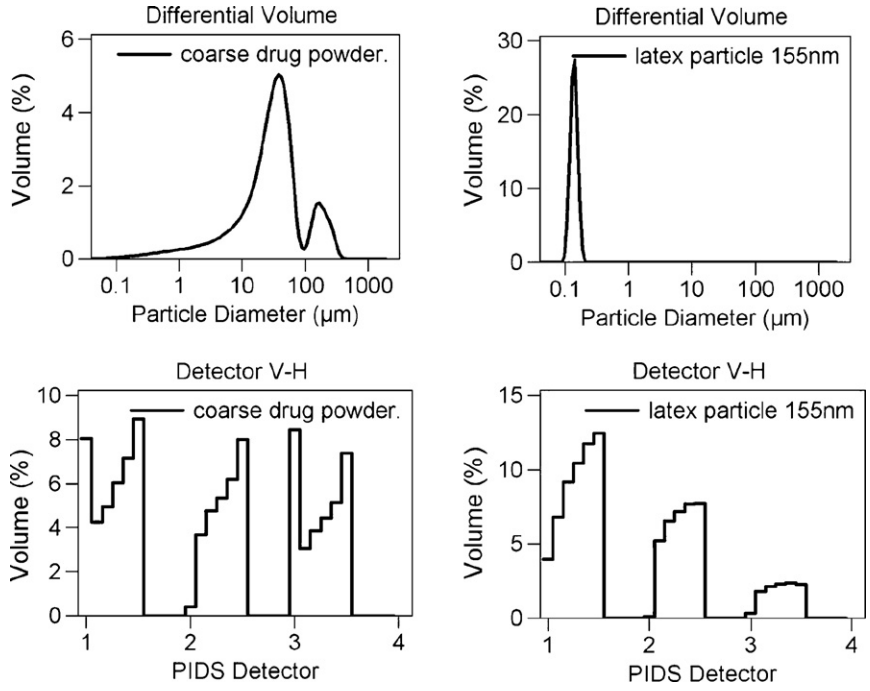

Fig. 7. Particle size distribution of coarse cyclosporine powder (40 μm) and latex particles (155 nm) (upper), and the corresponding PIDS data of the samples (lower). The three column groups represent the three different PIDS wavelengths (from left to right 450 nm, 600 nm and 900 nm), each of the three column groups consists of six columns, which represent the angles of detection (60–146°); the PIDS intensities of the coarse cyclosporine powder are nearly constant, whereas the intensities of the small latex particles change with a change in the wavelength.

to the right correspond to the light intensities recorded for the PIDS wavelengths 450 nm, 600 nm and 900 nm. Each column group consists of six columns, which correspond to the angles of record (60–146, from left to right). The figure clearly demonstrates the fact, that the scattering intensities of large particles are not or very little influenced by a change in the wavelength, whereas the small particles are strongly influenced.

The data obtained from PIDS measurements are directly incorporated into the data obtained from the pure LD measurement. The algorithm and how the data from the two different techniques are weighted and combined remains a manufacturer's secret.

In conclusion, modern laser diffractometers do not only rely on laser diffractometry measurements, but also use additional techniques to enhance the measuring range for submicron particles. The information obtained from the additional techniques is directly incorporated into the data obtained from the original LD measurement. Thus, it is important to realize: modern instruments provide results of two different technologies combined into only one result, which are falsely named LD measurement.

5. Influence on particle size and particle size distribution by changing the optical parameters

In order to understand to what extent the results are influenced by a change of the optical parameters, the influence due to changes of the real refractive index and the imaginary refractive index was investigated. This is possible, because the software of the LS 230 (and also of any other laser diffractometer) allows changes of the optical parameters also after the measurement itself. After changing the optical parameters the detected intensities (raw data) of the measurement are re-calculated by the software. The newly created results can be saved as a new file and compared between each other. For the experiment three different polydisperse latex dispersions were prepared (see Table 1) and analysed using the Fraunhofer approximation. PIDS technology was included in all measurements.

Afterwards the raw data of each measurement were re-calculated using various optical parameters (see Table 2). The newly created results were saved and compared between each other. The simulations of possible results were performed by combining every real part (RI) with every imaginary part (IRI) from the refractive indices selected in Table 2. For example, results were obtained by using the real refractive index of 1.6 for the analysis of the result, while this parameter was kept constant, the imaginary refractive index was varied (values used varied from 0 to 3, see Table 2). Thus, for every real refractive index 10 possible results, were obtained. In total 10 different real refractive indices were used for the simulations, leading to a total of 100 results per measurement. The simulations presented here, were obtained from the measurements of three different polydisperse latex dispersions (see Table 1). The results of the simulations are viewed in the next diagrams (Figs. 8 and 9). They show the changes of the parameters LD 50%, LD 90% and LD 100% analysed as volume distribution, which were obtained for the RIs 1.35, 1.4, 1.5, 1.6, 1.7 and 1.8 and for the IRIs 0, 0.01, 0.05, 0.1, 0.5, 1 and 3. The results obtained were plotted in two different ways. First the real refractive indices were plotted on the *x*-scale, leading to 6 main blocks. Within each main block the corresponding imaginary refractive indices were plotted (Fig. 8). Secondly the imaginary indices were plotted as main blocks and the real refractive indices were plotted within each main block (Fig. 9).

The simulations of the measurements clearly show the dramatic and unexpected high influence of the optical parameters on the analysis result. Changes in the optical parameters, not only influence the mean particle size (LD50) but also the particle size distribution (difference LD10 to LD100). Also there is no similarity in the simulation pattern between the three mixes investigated. It seems, as the changes in the result depend on the composition of the sample. However, by comparing the diagrams where the IRI was kept constant and the RI was changed (Fig. 9) a similarity can be seen. Obviously the impact of changing the real refractive index becomes less the higher the imaginary index is. However, in practice, in most of the cases samples have very small IRIs (IRI 0 to 0.1); hence the input of the IRI value has a great influence on the analysis result obtained.

In order to quantify the influence of the optical parameters on the result in Table 3 the smallest and the largest diameters and the resulting differences for the LD10 to LD100 are listed, again showing the tremendous differences. As example, the

Table 1
Composition of the latex dispersions analysed, standard particles were mixed to obtain multimodal distributions

	Particle size (μm)	Volume added (μl)[a]	Concentration in mixture (wt%)
Mix A tetramodal	0.102	8	18.2
	0.404	8	18.2
	0.845	20	45.2
	2.875	8	18.2
Mix B trimodal	0.404	8	40.0
	0.845	4[b]	20.0
	2.875	8	40.0
Mix B bimodal	0.204	20	94.3
	0.845	1.2[c]	5.7

[a] All standards had a particle concentration of 1 wt%.
[b] 20 μl, diluted 1:5.
[c] 6 μl, diluted 1:5.

Table 2
optical parameters used for the simulations, raw data were re-calculated combining each RI from the left column with all IRI from the right column

Real refractive indices (RI)	Imaginary refractive indices (IRI)
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

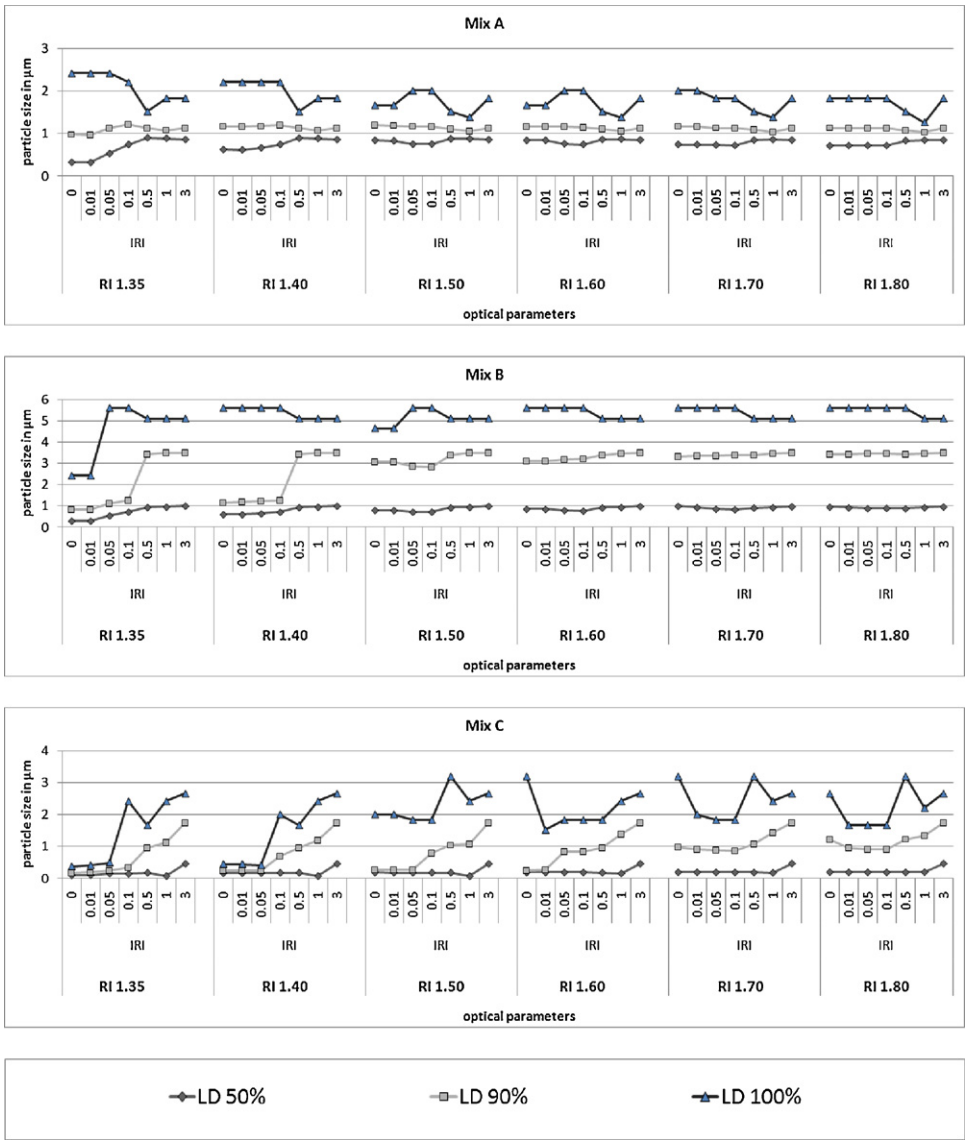

Fig. 8. Simulations of the latex mixtures: upper: Latex mix A, middle: latex mix B, lower: latex mix C. In each of the three graphs: The real refractive index (RI) was kept constant in each respective set, sets calculated for RI from 1.35 to 1.8 (left to right) and in each set the imaginary part (IRI) was varied from 0 to 3. The LD diameters 50%, 90% and 100% are plotted.

Table 3
Listing of minimal and maximal values and the resulting differences ($Difference_{max–min}$), obtained due to changes in optical parameters, shown for the diameters LD10, LD50, LD90, LD95, LD99 and LD100 (μm)

	Size in μm	LD10	LD50	LD90	LD95	LD99	LD100
Mix A	d_{min}	0.116	0.33	0.961	1.078	1.142	1.261
	d_{max}	0.745	0.905	1.223	1.468	1.636	2.423
	$Difference_{max–min}$	**0.629**	**0.575**	**0.262**	**0.39**	**0.494**	**1.162**
Mix B	d_{min}	0.106	0.284	0.842	1.067	1.435	2.423
	d_{max}	0.745	1.005	3.505	3.791	4.275	5.61
	$Difference_{max–min}$	**0.639**	**0.721**	**2.663**	**2.724**	**2.84**	**3.187**
Mix C	d_{min}	0.052	0.079	0.173	0.195	0.235	0.375
	d_{max}	0.154	0.465	1.744	1.883	2.342	3.206
	$Difference_{max–min}$	**0.102**	**0.386**	**1.571**	**1.688**	**2.107**	**2.831**

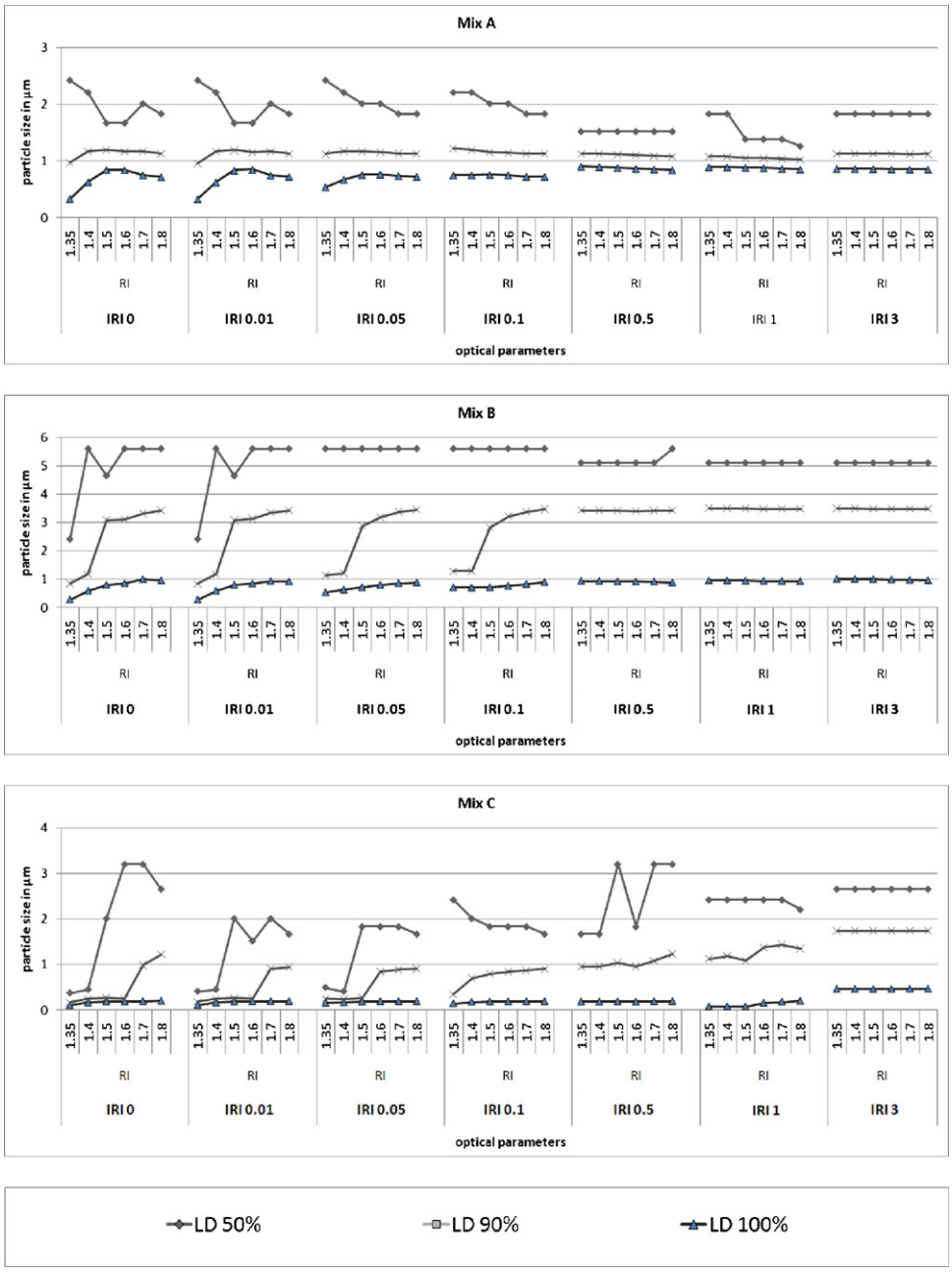

Fig. 9. Simulations of the latex mixtures: upper: Latex mix A (RI), middle: latex mix B, lower: latex mix C. In each of the three graphs: The imaginary refractive index (IRI) was kept constant in each respective set, sets calculated for IRI from 0 to 3 (left to right) and in each set the real part (RI) was varied from 1.35 to 1.8. The LD diameters 50%, 90% and 100% are plotted.

LD50 is chosen, often referred to be the mean particle size of a system. For Mix A the difference between the smallest and the largest LD50 simulated is 575 nm ($LD50_{min}$ = 330 nm, $LD50_{max}$ = 905 nm). Hence depending on the optical parameters used for analysis the result of the main particle size varies from only 330 nm to up to 905 nm. From this it is already clear, that laser diffractometry becomes useless for the characterization of submicron particles, if the optical parameters are not known, as the results will not correspond to the real particle size of the sample. However, it is not only the absolute particle size, but also the particle size distribution, which is of interest. Therefore, in the next diagrams, some results from the simulations were chosen and shown as size distributions curves (Figs. 10–12). Also here the raw data from one single measurement for each mixture were used to calculate the distribution curves for the different optical modules. The results clearly show that the distribution obtained strongly depends on the optical parameters used. Hence, no objective conclusion can be drawn about the size distribution without correct optical parameters. For example, Mix A (Fig. 10), in some cases the sample was analysed as monomodal, other simulation results give a bimodal or a trimodal distribution (seen as a weak shoulder in the graph, not separated from another fraction). In Mix B (Fig. 11) the results analysed vary from monomodal, bimodal and even tri-

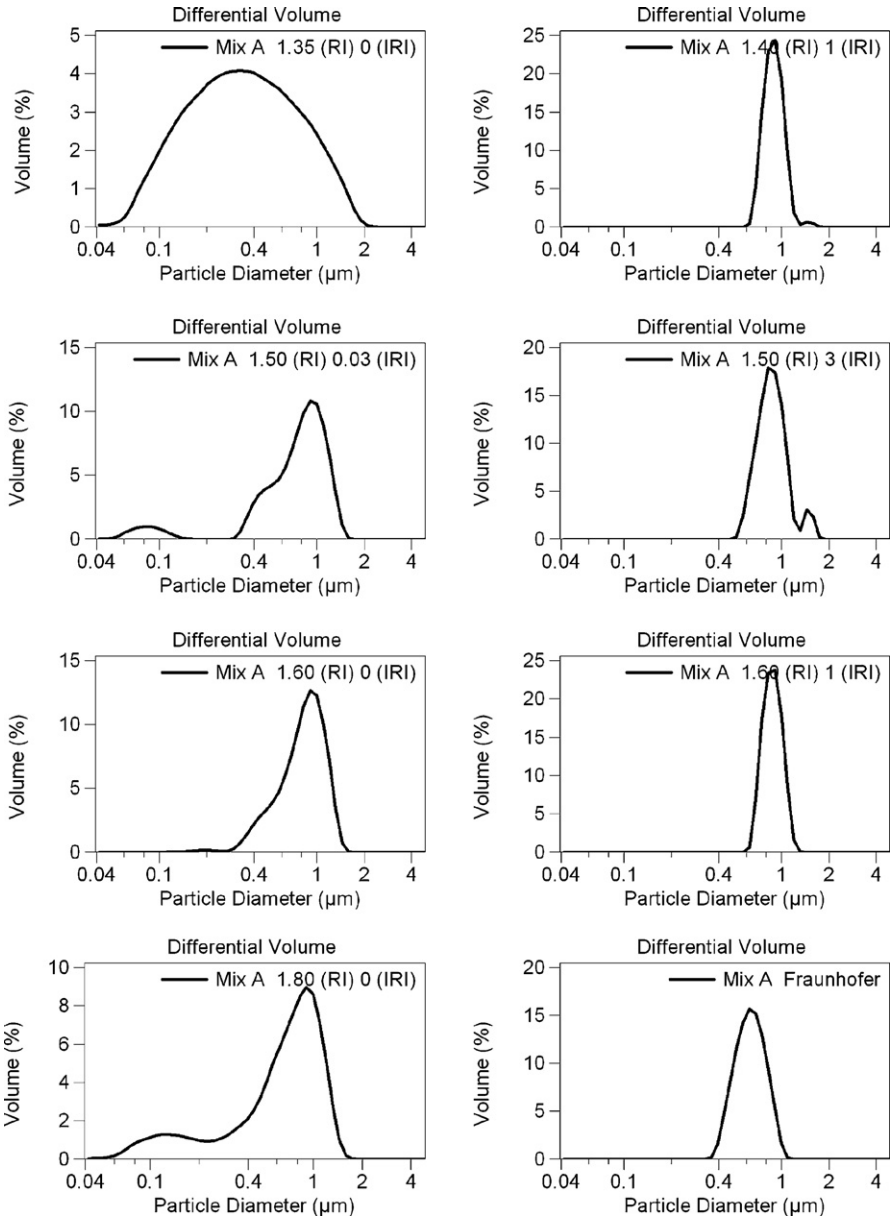

Fig. 10. Comparison of particle size distributions for the measurement of latex mix A analysed with different optical parameters. Depending on the optical parameters used, the distribution varies from monomodal to bimodal, from narrow to wide and detects smaller particles besides the main population of larger particles (in each distribution: in the upper right corner the RI (left) and IRI (right) used for analysis are given).

and tetramodal distributions and in Mix C (Fig. 12) even a pentamodal distribution was analysed.

In conclusion, the variation of the optical parameters enables the simulation of nearly every distribution, probably being far away from reality. Hence the use of incorrect optical parameters for analyses most likely creates false particle sizes and size distributions in reality.

6. Detection of larger particles besides a small sized main population

The previous results show the importance of the refractive index in respect to the result obtained. However, by screening the results critically one other important fact becomes obvious, especially if one looks at the results obtained for Mix A. Mix A is a tetramodal mixture, containing particles with a size of 100 nm (18.2%), 400 nm (18.2%), 845 nm (45.5%) and a larger fraction of 2875 nm (18.2%) (see Table 1). However, the largest particle population of 2.875 μm is totally missing in most of the simulation results (e.g. values of LD99 and 100 do not exceed values higher than 1 μm—see Fig. 6). This finding indicates that LD measurements may fail to detect larger particles besides a small sized main particle population, which is well in agreement with the work of Driscoll et al. (Driscoll et al., 2001), see 1). Therefore, to prove the usefulness of the LD instrument, samples consisting of a small sized main population and larger particles were analysed. The data shown here were obtained from a physically non-stable tretinoin loaded NLC suspension, hence

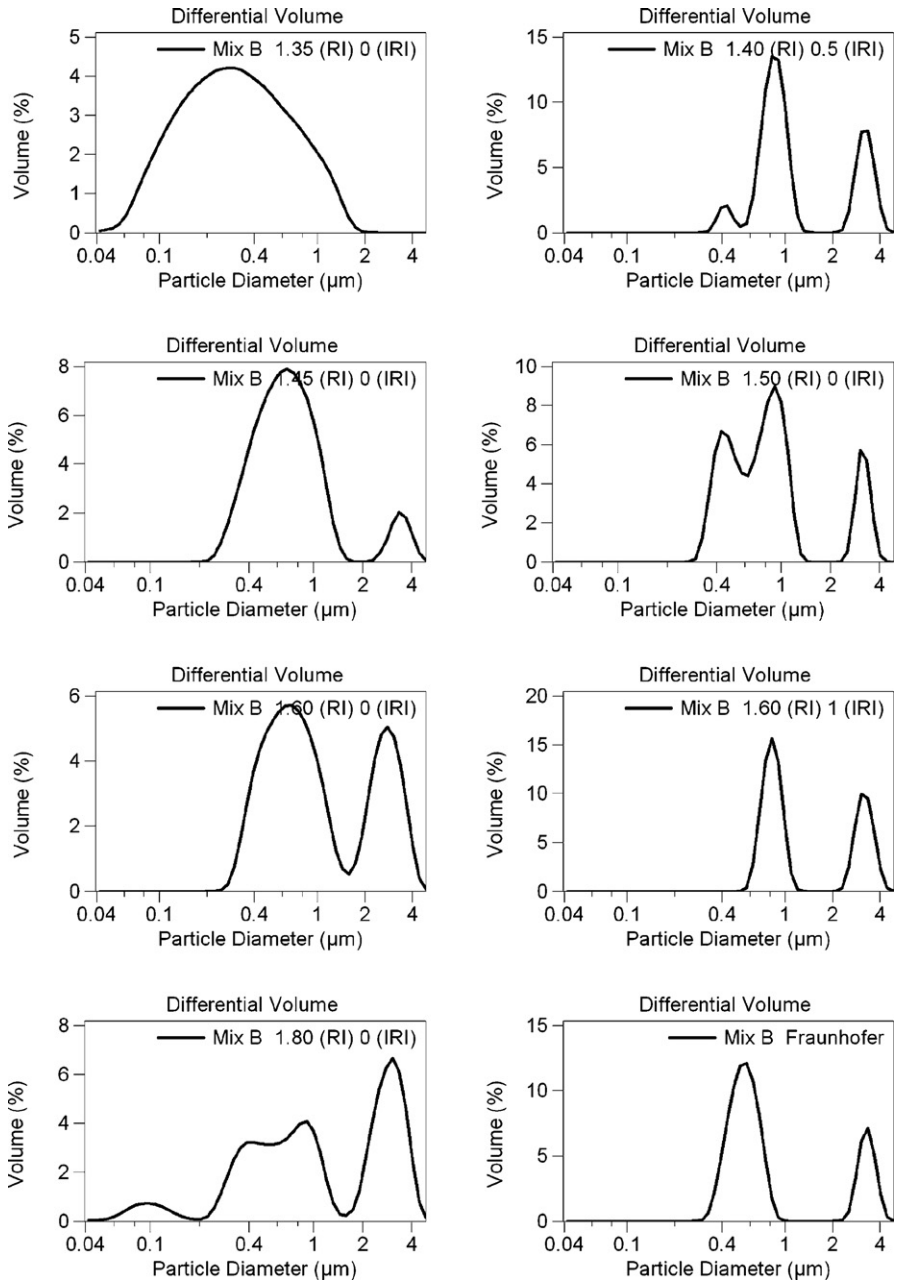

Fig. 11. Comparison of particle size distributions for the measurement of latex mix B. The results vary from monomodal, to bimodal and trimodal and even a tetramodal distribution can be analysed.

it consisted not only of small lipid nanoparticles, but also contained some medium sized particles (approx. 1.5 μm) and many large tretinoin crystals (sizes ranging from 10 μm to 50 μm), as seen from polarization microscopy (Fig. 13).

The analysis result of the LD measurement, which was performed as suggested from the handbook of the instrument (included PIDS, Fraunhofer analysis), is shown in Fig. 14. The main particle size was analysed to be 1.09 μm (LD50) and no particles larger than 3.2 μm (LD100) were found when Fraunhofer analysis mode was used for the calculation. Thus, the assumption, the LS 230 might oversee larger particles was proven.

Of course, as seen in Section 5, the influence of the optical parameters is tremendous. Therefore, to investigate, if the failure in the detection of the larger particles is due to the incorrect input of the optical parameters, the raw data of the measurement were simulated as seen in the previous chapter. Within this simulation the LD100, representing the largest particles in the system, never exceeded sizes above 3.5 μm, clearly indicating that no larger particles are detected, independent on the optical parameters used. Hence the instrument overestimates the presence of small particles and overlooks larger particles (e.g. >5 μm). From these results (other data not shown) one could easily draw the conclusion: laser diffractometry really fails for the detection of large particles besides a small sized main population. With that, the work of Driscoll et al. would be proven, clearly showing that LD fails its primary intend and is not useful at all.

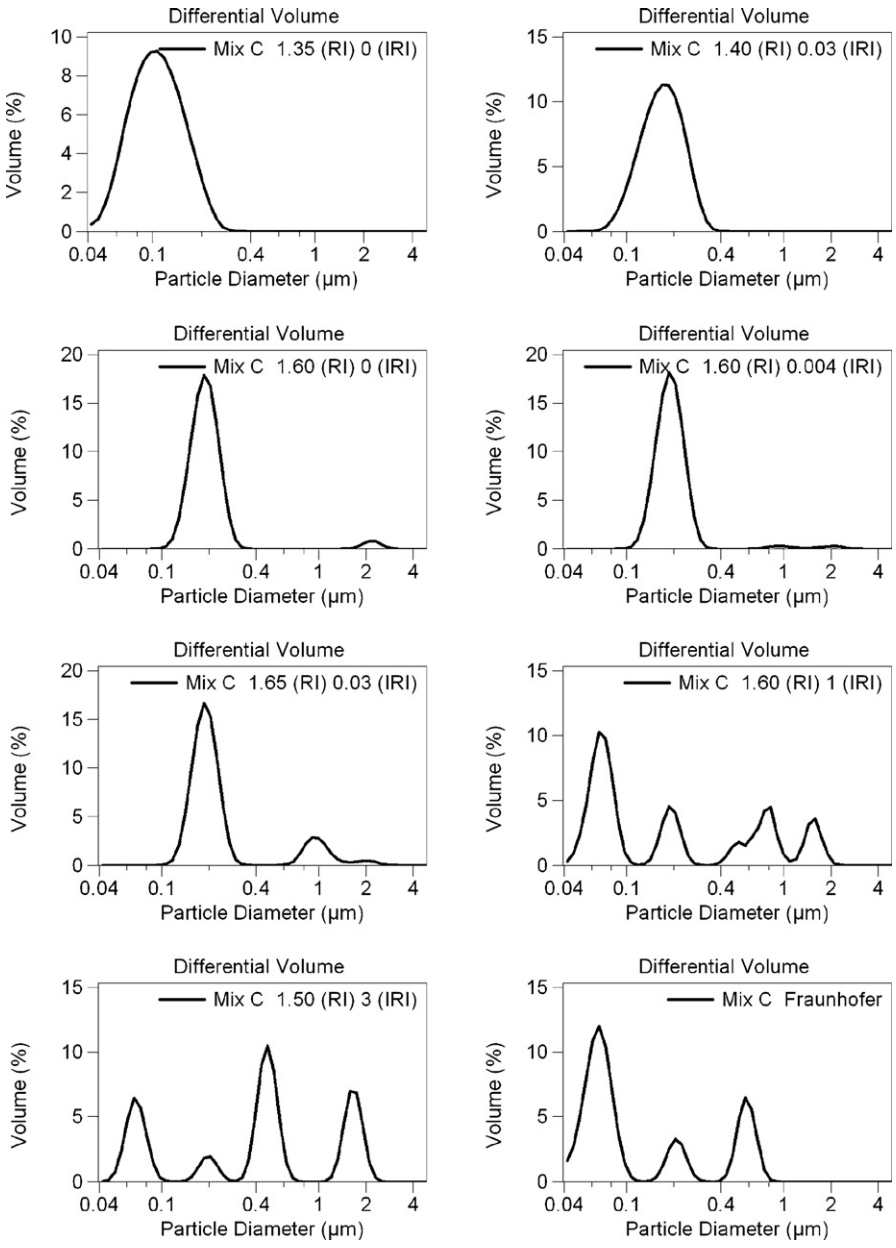

Fig. 12. Comparison of particle size distributions for the measurement of latex mix C. The results vary from monomodal, to pentamodal, even if the changes in the optical parameters are very little (e.g. change only IRI from 0.01 to 0.3 and constant RI).

However, by taking one further step and thinking about the method and the setup of the instrument, the undesired result might be explained as follows: as mentioned above, the instrument does not only measure the diffraction pattern of the sample, but also uses an additional technique to extent the submicron measuring range (in case of the LS 230 it is PIDS, but is different in other instruments). For that the instruments consist of two different sample cells (PIDS cell and LD cell). For a measurement the sample is added to the dispersing unit and pumped through the sample cells, which are placed underneath each other. There are detectors behind both cells to detect the obscuration (as a measure of the sample concentration) in the cells. If submicron particles are analysed the additional PIDS technique must be used for the enhancement of the submicron measuring range, thus if the sample is added to the dispersion unit, the obscuration in the PIDS cell is a measure for the correct sample amount added into the instrument for analysis. The obscuration reached should be in the range between 40% and 50%. However, if no submicron particles are analysed, PIDS technology does not need to be included. In such cases the obscuration of the LD cell and not the PIDS cell is the measure for the correct sample amount and should be in the range between 7% and 12%. Interestingly, when PIDS technology is used, not only the actual obscuration of the PIDS cell, but also the obscuration of the LD cell is viewed on the monitor at the same time. This feature gives the possibility to compare these two obscurations directly. In case of the sample investigated here, the PIDS obscuration was 45% and the LD obscuration was only 1%. Hence the PIDS

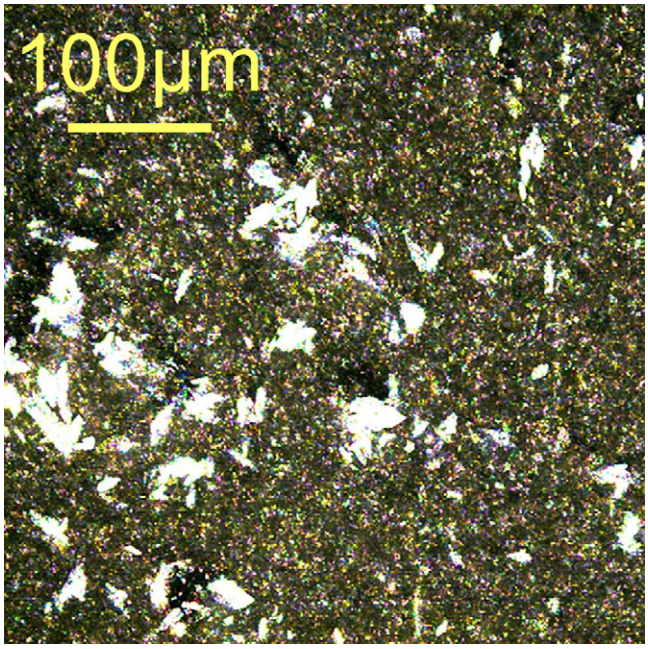

Fig. 13. Microscopic image with polarized light of the tretinoin nanoparticle suspension (magnification 160×). As seen from the micrograph, the system also contains different fractions of larger tretinoin crystals with sizes of 2 µm, 10 µm and very large particles between 50 µm and 100 µm.

obscuration was sufficient high for the measurement, but the LD obscuration (measuring larger particles) was too low. Therefore, it was expected from the theory of the technique, that if the same sample is measured again, but with a sufficiently high LD cell obscuration, the larger particles should be detected.

Thus, the sample was analysed again without PIDS technology and with a LD cell obscuration of 12%. The results of both, this analysis (without PIDS) and the previous result with included PIDS are viewed in Fig. 15.

As expected from the theory, large crystals were detected when the correct LD obscuration was used for analysis. The result emphasizes two facts:

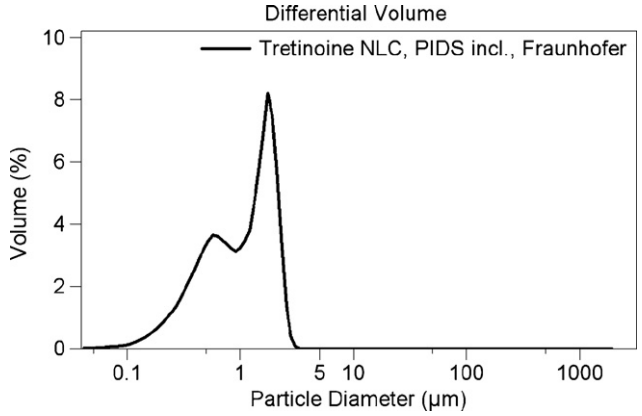

Fig. 14. LD data (Fraunhofer analysis) of the tretinoin loaded lipid nanoparticle suspension known to contain large particles (see Fig. 13). The measurement was performed as suggested for samples containing submicron particles. Unfortunately the larger particles are not detected at all.

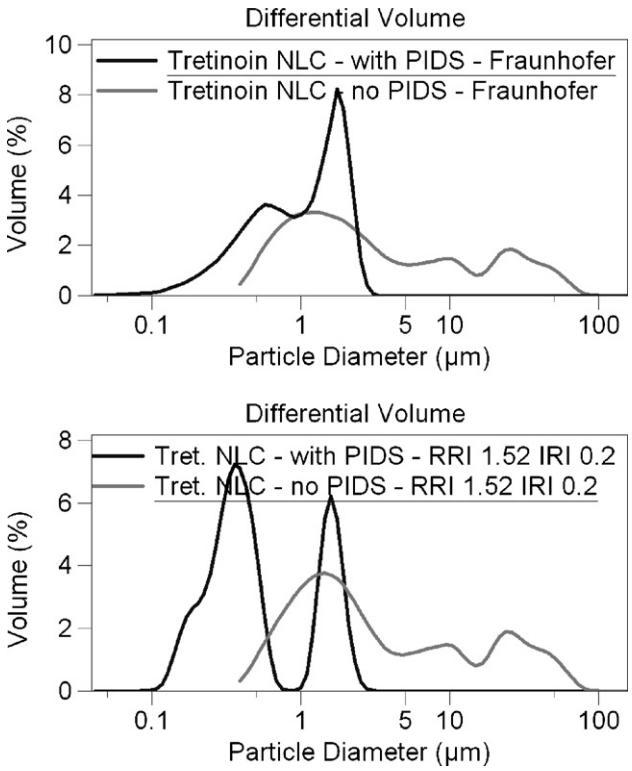

Fig. 15. Influence of the PIDS technology on the analysis result: comparison of LD data from measurements with and without PIDS (left: Fraunhofer analysis, right: Mie modus with the correct optical parameters 1.52 for RI and 0.2 for IRI; blue line with included PIDS technology, green line: without PIDS technology): measurements with included PIDS technology do not detect larger particles in the system, the problem can be overcome by excluding the PIDS technology from the measurement, performing a simple LD measurement.

1. additional techniques, different to LD, can be very problematic to the usefulness of LD measurements, as they overestimate the presence of small particles and consequently fail to detect few large particles,
2. the problems can be overcome by simply excluding these techniques from the measurement and preferring a pure LD measurement (with simple Fraunhofer analysis for particles >6λ and Mie analysis with correct optical parameters for particles <6λ (λ = wavelength of laser).

7. Conclusion

In the last years the world's interest in nanoparticles increased and with that the need to have methods for analyzing these nanoparticulate systems. Manufacturers of LD instruments fulfilled the costumers need and increased the measuring ranges of their instruments down to the lower submicron range. Clearly with that a former simple method became more complicated. Thus more knowledge and understanding of this method is necessary to perform correct measurements. Unfortunately this is often overseen by the consumers and at the other hand not often sufficiently emphasized by the manufacturers. The paper clearly proves that laser diffractometry becomes useless, i.e. meaningless results are obtained, if the technique is not used appropriately. Optical parameters, often referred to be an unim-

portant parameter for LD results, were doubtlessly proven to be very crucial. Changes in these parameters change the particle size and the particle size distribution tremendously. Larger particles might be overlooked if small particles are in the sample and if the analysis is performed in the mode especially created for the measurement of small particles. Therefore, Driscoll et al. are right in the fact that laser diffractometry can be misleading, but from the experience of our work, only if the technique is used inappropriately. The paper provides evidence, that these errors can be easily overcome, leading to correct results. Thus, we state that if the technique is used without sound knowledge, there is a great danger that the analysis and the interpretation of these data will lead to artificial results and to false interpretations and conclusions therefore. Thus, the overall conclusion is every operator using laser diffractometry should understand the method, before starting a measurement. Also any result and any publication showing LD data without the information of the optical parameters and/or without the measuring parameters used, as well as data which were analysed or using guessed optical parameters must be doubted. This is probably true for at least 90% of all the data published for submicron particles.

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