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Taking Subvisible Particle Quantitation to the Limit: Uncertainties and Statistical Challenges With Ophthalmic Products for Intravitreal Injection



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ABSTRACT

Subvisible particles are a critical quality attribute of pharmaceutical products. The reliability of particle quantitation increases with the number of particles in the analyzed sample volume. However, for analyses of low-volume drug products, such as ophthalmic products for intravitreal injection or biopharmaceuticals in general, sample volumes as small as possible should be used to avoid pooling and consequently, the contamination with foreign particles. The aim of our study was to evaluate the variability of particle concentrations obtained by light obscuration measurements to define the minimum required analyzed sample volume to achieve statistically meaningful results by using conditions that are practically feasible. Statistical evaluation suggests that for particle concentrations close to a predefined limit, large sample volumes (a multiple of typical intravitreal product volumes) would be required for a high probability to correctly classify samples with respect to the predefined limit. Below a minimum analyzed volume, even a measurement result of 0 particles does not allow to conclude compliance with the respective particle concentration limit with sufficient certainty. A small analyzed volume could be justified as long as the measurement uncertainty remains acceptable compared with the predefined limit.

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Introduction

Particles in biopharmaceuticals can originate from many sources and can, among other descriptors, be classified by their origin as inherent, intrinsic, or extrinsic.^{1,2} Proteins in biopharmaceuticals can aggregate and thus form inherent particles of different sizes, which might have an impact on product safety, efficacy, or pharmacokinetics.^{3–5} Nonproteinaceous particles can be introduced during manufacturing and processing of a drug, from the primary packaging or from formulation components, for example, broken glass, abraded rubber, metal, or cellulose fibers, silicone oil droplets from prefilled syringes, fatty acid particles from degraded polysorbate, or delaminated glass.^{6–10} Therefore, the presence or

absence of particles are important indicators of purity and stability of a biopharmaceutical drug product, and they are considered as critical quality attributes which have to be monitored throughout the entire product lifecycle. However, the biological consequence of particles and amounts thereof in biopharmaceutical products is beyond the scope of this article.

Currently, particle quantification is mainly focused on subvisible particles in the size range of 10–100 µm where particle number limits are defined by the Pharmacopoeias. Several analytical methods are available for the quantification of subvisible particles. The Pharmacopoeias allow the usage of light obscuration (LO) and microscopy. Particle concentration limits for LO are discussed in this article. For the microscopy method, readers are referred to the respective chapters of the Pharmacopoeias. Specifically, USP <788> “Particulate matter in injections”¹¹ (harmonized with Ph. Eur. 2.9.19¹²) requires preparations of less than 100 mL to contain no more than 6000 particles ≥ 10 µm per container and not more than 600 particles ≥ 25 µm per container as determined by LO; preparations of more than 100 mL are required to contain no more than 25 particles/mL ≥ 10 µm and no more than 3 particles/mL ≥ 25 µm.¹¹

Abbreviations used: DP, Drug product; IVT, intravitreal injection; LO, light obscuration; RSD, relative standard deviation.

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Products for intravitreal injection (IVT products) are a special case for which USP <789> "Particulate matter in ophthalmic solutions"¹³ applies. USP <789> requires ophthalmic solutions to contain not more than 50 particles/mL $\geq 10\ \mu\text{m}$, not more than 5 particles/mL $\geq 25\ \mu\text{m}$, and not more than 2 particles/mL $\geq 50\ \mu\text{m}$.¹³ These low limits are very challenging in terms of statistically relevant analysis results, in particular with the small volumes of typical IVT products ($<1\ \text{mL}$).¹⁴ Appropriate analyzed sample volumes should therefore be chosen. It should be noted that USP <789> does not have a counterpart in other pharmacopeia.

The minimum sample volume required for the test according to USP <788> is 25 mL.¹¹ This is not appropriate for many biopharmaceutical products, including IVT products, with extractable volumes below 1 mL owing to the necessity of extensive pooling to obtain the required sample volume of 25 mL for the particle count test. For example, assuming a typical sample volume of 100–200 μL , pooling of 125–250 units would be required for 1 measurement. Apart from possible masking of container-to-container variability^{15,16} and increased material demand and sample preparation time, pooling may introduce additional particles through external contaminants and aggregation of the protein during handling. For such cases, USP <787> "Subvisible particulate matter in therapeutic protein injections"¹⁷ allows smaller test volumes down to approximately 1 mL. The general feasibility of methods for subvisible particle counting with analyzed volumes as low as allowed by USP <787> is reported in literature.^{15,18} However, statistical relevance of results derived from low-volume methods in the particle concentration range which is relevant with respect to USP <789> has not been considered, yet.

From the statistical point of view, the distribution of particles or any other analyte in a sample can be assumed to be based on Poisson distribution,^{16,18,19} which is applicable for the counting of discrete events. Even though this statistical counting model is well-known, it is unclear, to what extent it is applicable for LO data, because the uncertainty of final measurement results will be additionally influenced by sample preparation and instrument accuracy. Experimentally, the precision of subvisible particle counting was recently reported to be independent of particle size but strongly dependent on the particle number in the analyzed volume.¹⁶ Additionally, a lower limit of the counting capability of LO of 50–500 particles/mL (dependent on the type of particles) was estimated based on observed overcounting of proteinaceous particles by approximately 200%–500% for theoretical particle concentrations of approximately 100 particles/mL.²⁰ This suggests that accurate determination of particle concentrations on the order of the limits required by USP <789> is difficult.

The aim of our study was to evaluate the uncertainty of LO measurements at low particle concentrations, relevant for typical IVT products, to find an appropriate measurement volume (statistically meaningful and practically feasible). Specifically, the probability distribution underlying the experimental data was assessed for suspensions of polystyrene standard beads. Even though polystyrene particles differ from protein particles in terms of physical properties, for example, refractive index, they are a good model to assess the general distribution of low particle numbers in a defined sample volume. They represent a best-case scenario regarding the detection by LO, because the sample-to-sample variability is low. This enables the theoretical evaluation of the probability to distinguish different particle concentrations based on statistical significance. Furthermore, the probability to correctly rate a sample as being below or above a predefined limit and the confidence intervals of potential measurement results were evaluated. Based on these considerations, method parameters such as analyzed volume and number of replicate analyses can be defined to deal with the large uncertainty in the data.

Materials and Methods

Materials

Purified water (Milli-Q IQ7000 or Milli-Q Advantage A10, Merck Millipore, Darmstadt, Germany) with a resistivity of $>18\ \text{M}\Omega\ \text{cm}$ and sterile filtered (PVDF membrane with $0.22\ \mu\text{m}$ pore size, Merck Millipak) was used for all experiments exclusively and is termed "water" in the following. Polystyrene standard particle suspensions (Duke standards 4K-10, 4K-25, and 4K-50) were purchased from Thermo Scientific (Fremont, CA). Triton X-100 (BioXtra) was purchased from Sigma-Aldrich (Munich, Germany).

Dilution of Stock Suspensions With Polystyrene Standard Beads

A filtered ($0.22\ \mu\text{m}$) 0.02% (v/v) solution of Triton X-100 in water was used as diluent when diluting stock suspensions of polystyrene standard beads. The stock suspensions of polystyrene beads were resuspended by 3 min vertical rotation at 10 rpm followed by 1 min sonication at an ultrasonic peak output of 480 W (Bandelin Sonorex Super RK 106, Bandelin electronic GmbH, Berlin, Germany) directly before dilution. Sterile media bottles made from polyethyleneterephthalate-G-copolymer (Nalgene; Thermo Fisher Scientific, Waltham, MA) or polypropylene tubes (Greiner Bio-One, Kremsmünster, Austria) were used as containers for the dilutions. Dilutions with nominal concentrations of 6 particles/mL, 60 particles/mL, and 600 particles/mL were prepared based on the approximate particle concentration of the stock suspensions given by the manufacturer. The suspension with 600 particles/mL was prepared directly from the stock suspension. Suspensions at 60 particles/mL and 6 particles/mL were prepared by 10-fold and 100-fold dilution, respectively, of the 600 particles/mL suspension. All preparation steps, except for resuspension and pipetting of polystyrene stock suspensions, were performed in a laminar air flow environment. Each suspension was slowly inverted 20 times directly after preparation. Suspensions were prepared and stored at room temperature and were used within 2 days after preparation.

Light Obscuration Measurements

The diluted polystyrene bead suspensions were vertically rotated for 2 min at 10 rpm before each sample withdrawal for measurement. Samples were then transferred to, washed and dried, 20-mL or 2-mL glass vials (FIOLAX, Schott, Mainz, Germany) in a laminar air flow environment.

The PAMAS SVSS-C Sensor HCB-LD-25/25 (Partikelmess-und Analysensysteme GmbH, Rutesheim, Germany) was used for LO measurements.

Each LO measurement consisted of a pre-run (not included in data evaluation, particle concentration not recorded) and 10 sub-runs. Twenty replicate measurements were performed for each sample. Measurements were carried out on 2 different sample volume scales. For 10-mL measurements, a pre-run volume of 0.5 mL sample was followed by 10 sub-runs of 1 mL each. For 1-mL measurements, a pre-run volume of 0.2 mL sample was followed by 10 sub-runs of 0.1 mL each. Ten milliliter measurements were performed with 10- μm particles at nominal concentrations of 60 and 600 particles/mL, with 25- μm particles at nominal concentrations of 6, 60, and 600 particles/mL, and with 50- μm particles at nominal concentrations of 6 and 60 particles/mL. One milliliter measurements were performed with 25- μm particles at nominal concentrations of 6 and 60 particles/mL.

To prevent sedimentation of the particles, 2 min horizontal orbital shaking (IKA MS 3 basic shaker, IKA-Werke GmbH & Co. KG, Staufen, Germany; equipped with a customized sample holder) at

approximately 200 rpm was applied to the vial with the sample before the measurement was started. The same shaking was applied during measurements. For the measurements of 50- μm particles, shaking was performed at a speed of 300 rpm and was stopped before the ninth sub-run to prevent intake of air.

Cumulative particle numbers were recorded for the following particle diameters: ≥ 1.0 , 2.0, 3.0, 5.0, 7.5, 8.3, 10.0, 15.0, 20.0, 25.0, 30.0, 50.0, 70.0, 75.0, 100.0, and 200.0 μm . For the evaluation of the concentration of polystyrene standard beads with nominal diameters of 10 μm , 25 μm , and 50 μm , respectively, the concentrations of particles in the size ranges 7.5–15 μm , 20–30 μm , and 30–70 μm , respectively, were extracted from the data (for size distributions of the particle standards see the supplementary information, Fig. S1). Mean particle concentrations calculated from individual sub-run results are provided with 1 decimal digit in the results part to allow traceability of subsequent calculations.

After each sample measurement, the measurement cell and tubing of the LO instrument were rinsed with at least 10 mL water, and a control measurement of water was performed to confirm the cleanliness of the LO instrument. Washing was repeated until no particles ≥ 8.3 μm and less than 50 particles/mL in total were present.

Theoretical Calculations

Probabilities and distributions were calculated by using the software environment R.²¹ Calculations of probabilities are based on the assumption that measurements of particle numbers in the considered particle number range follow a Poisson distribution, which was confirmed by the supporting experiments with polystyrene standard beads.

Example calculations for illustration purposes are mainly derived from the USP <789> limit for particles ≥ 50 μm . Specifically, a predefined limit of 2 particles/mL was used as threshold for the classification of hypothetical measurement results as below (≤ 2 particles/mL) or above (> 2 particles/mL) the limit.

Power Analysis

The power analysis for the differentiation of 2 different particle concentrations was based on a significance level α of 5%. From that, a critical value was calculated which corresponds to the $(1-\alpha)$ quantile of the distribution centered at the lower concentration. The power resulted as the cumulative probability density above the critical value of the distribution centered at the higher concentration (Fig. 3e).

Probability of True-Positive and False-Positive Results

A true-positive result in terms of this study was defined as the finding of a particle concentration above a predefined limit when the true particle concentration in the sample is larger than the predefined limit (i.e., true “fail” result). False-negative results (i.e., false “pass” results) occur at a probability of $(100\% - \text{probability of true-positives})$. Similarly, a false-positive result was defined as the finding of a particle concentration above the predefined limit when the true particle concentration in the sample is smaller than the predefined limit (i.e., false “fail” result). True-negative (i.e., true “pass” results) can be expected at a probability of $(100\% - \text{probability of false-positive results})$ (Fig. 4c).

For the calculation of the probabilities, a limit particle concentration was defined, at which a measurement result would be rated as “fail.” This limit concentration was set to “predefined limit +0.5 particles/mL” (lowest concentration that would be rounded up to a value above the predefined limit when obtained as

an average measurement result). For example, at a predefined limit of 2 particles/mL, measurement results of ≥ 2.5 particles/mL were rated as “fail.” Limit particle concentrations were converted to limit particle numbers for different analyzed volumes. Noninteger limit particle numbers were rounded up to the next larger integer to account for the restriction that Poisson-distribution based probabilities can only be calculated for integer quantiles. Expected average particle numbers for different analyzed volumes and for different true particle concentrations in a sample were compared with the limit particle number. Particle numbers were rated as “fail” if they were larger than or equal to the limit particle numbers.

Calculation of Confidence Intervals

To assess the probability that the true particle concentration in a sample complies with a predefined limit for different hypothetical measurement results, confidence intervals were calculated. As concentration limits are usually upper limits, only the upper limits of one-sided confidence intervals are of interest. The upper limit of the one-sided $(1-\alpha)$ confidence interval, $\lambda_{u, \text{Poisson}}$, for Poisson-distributed variables is calculated by using the quantiles of the χ^2 -distribution (Eq. 1).^{22,23}

$$\lambda_{u, \text{Poisson}} = \frac{1}{2N} \chi^2_{1-\alpha; 2k+2} \quad (1)$$

In Equation 1, k is the sum of the number of particles from N hypothetical measurement results. The upper confidence interval limits at different confidence levels for a hypothetically measured particle number were compared with a particle number limit k_{max} which was calculated from the predefined particle concentration limit and a given analyzed volume. k_{max} was rounded down to the nearest integer to avoid overestimation of the achievable confidence level (rounding was necessary owing to the restriction of the Poisson distribution to integer quantiles). From that, the maximum possible confidence level for a given combination of measured particle concentration and analyzed volume was derived as the highest confidence level for which $\lambda_u \leq k_{\text{max}}$.

Results

The distribution of measurement results in particle quantitation should in theory be based on a combination of (1) the Poisson distribution, which accounts for the distribution of the particles within the sample,^{16,18,19} with the addition of (2) an unknown (experimental) component, which can be further divided into influences from sample preparation and from instrument specific uncertainties. The former can be assessed by theoretical calculations, whereas the latter, and thus the total method variability, can only be deduced from experimental data. In this section, results from the assessment of experimental variability are presented. Based on these results, probabilities of different outcomes of particle quantitation analyses are evaluated.

Experimental Assessment of the Counting Uncertainty in LO Data

Data sets of LO measurement data of polystyrene standard beads were generated for particle diameters of 10 μm , 25 μm , and 50 μm and nominal particle concentrations of 6, 60, and 600 particles/mL.

The relative standard deviation of the particle concentrations obtained from 1.0-mL sub-runs was 5%–7% at a nominal concentration of 600 particles/mL and 47%–58% for 6 particles/mL, independent of the nominal particle size (Table 1). Thus, the variability increased with decreasing particle concentration. The considerable

Table 1
Summary of LO Results for Polystyrene Standard Beads

Nominal Concentration [#/mL]	Nominal Particle Diameter [µm]	Mean of All Sub-runs [#/mL]	Standard Deviation of All Individual Sub-runs [#/mL]	Relative Standard Deviation of All Individual Sub-runs [%]	Minimum Concentration in 1 Sub-run [#/mL]	Maximum Concentration in 1 Sub-run [#/mL]
6	25	4.8	2.3	47	1	12
	50	3.5	2.0	58	0	9
	10	58.0	8.5	15	37	84
60	25	47.9	7.8	16	31	72
	50	37.9	10.3	27	17	74
600	10	536.5	24.5	5	481	597
	25	462.4	34.6	7	367	555

For each combination of particle size and concentration, 20 measurements with 10 sub-runs (200 sub-runs in total) of 1.0 mL each were performed.

variability of approximately 50% at a nominal concentration of 6 particles/mL can be explained based on the assumption of Poisson distribution of particle counts. The relative standard deviation of a Poisson distribution with a mean of 6 would be 41% ($1/\sqrt{6}$); for the measured means of 3.5 and 4.8 particles/mL, the relative standard deviation predicted by Poisson statistics would be 53% and 46%, respectively. At 600 particles/mL, the standard deviation of the particle concentration in the experimental data tended to be larger than that predicted by a Poisson distribution ($1/\sqrt{600} = 4\%$ and $\sqrt{600} = 24.5$ particles/mL).

The particle concentrations resulting from 0.1-mL sub-runs showed larger variability than the data from 1.0-mL sub-runs (Fig. 1). For example, at a nominal concentration of 6 particles/mL, the standard deviation of the particle concentration reached 117% (6.8 particles/mL) in data from 0.1-mL sub-runs in contrast to 47% (2.3 particles/mL) in data from 1.0 mL sub-runs. The larger variability with 0.1-mL sub-runs can be explained by the conversion from measured particle numbers per sub-run (<1 mL) to particle concentrations per mL. A similar observation has been reported for the quantification of particles in the nanometer range by resonant mass measurement and nanoparticle tracking analysis in even smaller sample volumes.¹⁶ Specifically, in 0.1 mL analyzed volume, 1 additional particle, for example, due to contamination or instrumental fluctuation, would be converted to additional 10 particles/mL, whereas the same additional particle in 1 mL analyzed volume would be converted to 1 particle/mL.

For a nominal concentration of 6 particles/mL, an average particle number of 0.6 is expected in each sub-run of 0.1 mL. The probability to encounter at least 1 particle in 1 sub-run is in this case 45% (based on the assumption of Poisson distribution). Experimentally, particle numbers per 0.1-mL sub-run of 0, 1, 2, or 3 were observed and were converted to particle concentrations between 0 and 30 particles/mL.

Importantly, the mean and standard deviation of particle numbers from a total analyzed volume of 1.0 mL were independent of how the total analyzed volume was collected. The summation of particle numbers from 10 sub-runs of 0.1 mL each yielded the same results as a single determination with a corresponding total volume (Table 2, compare second and third line). This shows that sub-runs with a volume of less than 1.0 mL (e.g., to reduce pooling risks by minimizing the sample volume per pool) is possible when the total analyzed volume of all sub-runs amounts to at least 1.0 mL. In contrast, conversion of particle numbers to particle concentrations for individual sub-runs of <1.0 mL should be avoided, because this leads to higher standard deviations (Table 2, first line).

To find an appropriate model for the distribution of measurement results, theoretical distributions (based on Poisson and normal distribution) of particle concentrations were calculated and were compared with the experimental data. The Poisson distribution can describe the distribution of discrete numbers, for example, the particle distribution throughout the sample volume for properly homogenized samples. The normal distribution describes continuous data; for example, the distribution of measurement data when experimental uncertainties, such as sample preparation, have a larger impact than the statistical distribution of particles in the samples.

The fit of the distributions was assessed for the 3 considered particle concentration levels (6, 60, and 600 particles/mL) separately. For 1.0-mL sub-runs, data from the different particle sizes (10 µm, 25 µm, and 50 µm, see also Table 1) were combined. Mean and standard deviation of the respective data sets were calculated. Quantiles of Poisson distributions with the same mean and of normal distributions with the same mean and standard deviation as in the experimental data sets were calculated. For the quantile-quantile-plots in Figure 2, particle numbers in each experimental data set

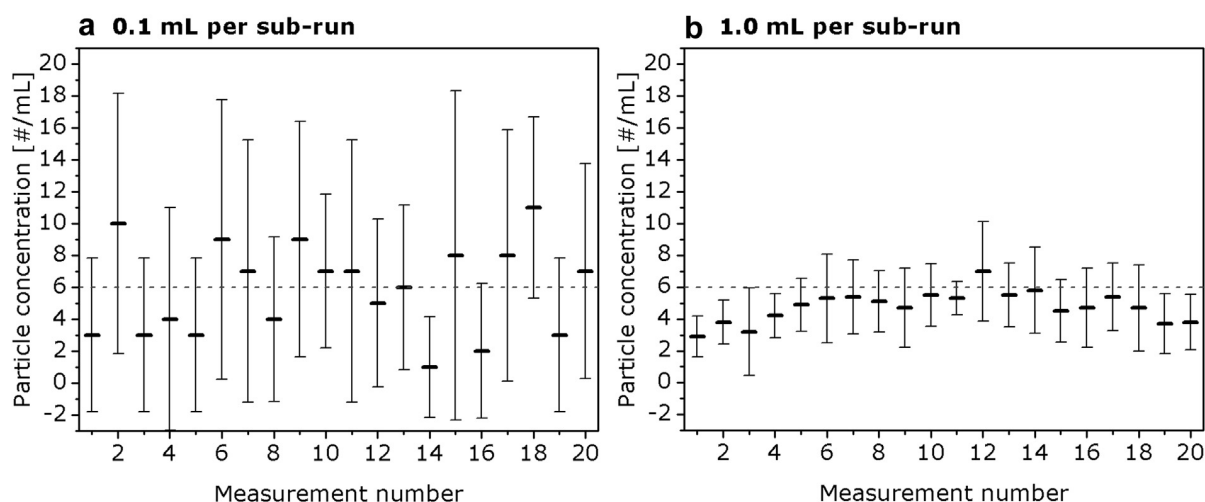


Figure 1. Concentration of particles $\geq 20 \mu\text{m}$ and $< 30 \mu\text{m}$, measured by LO in a sample with $25\text{-}\mu\text{m}$ polystyrene standard beads at a nominal concentration of 6 particles/mL. Mean and standard deviation of 10 sub-runs each from 20 independent measurements. (a) Sub-runs of 0.1 mL, (b) sub-runs of 1.0 mL.

were sorted in increasing order and were compared with the quantiles of the 2 theoretical distributions. Each point in the graphs represents the measurement result of 1 sub-run. If the theoretical distribution is appropriate for the description of the experimental data, the points will follow a straight line through origin.

The comparison showed that the Poisson distribution was appropriate to describe experimental data from 1.0-mL sub-runs at a particle concentration of 6 particles/mL (compare Figs. 2a and 2b). For particle concentrations of 60 particles/mL and larger, a normal distribution appeared to be more appropriate to describe data from 1.0-mL sub-runs (Figs. 2c-2f).

Distributions of data from 0.1-mL sub-runs were evaluated based on particle numbers which were not converted to particle concentrations to avoid the large uncertainty induced by conversion (Fig. 1). For a particle concentration of 6 particles/mL, the measured particle numbers per sub-run were 0, 1, 2, or 3 (Figs. 2g-2h). Such a small number of different measurement results was in accord with the expected average number of 0.6 particles per 0.1-mL sub-run for 6 particles/mL, but it does not allow to estimate a distribution. For a nominal concentration of 60 particles/mL, that is, an average expected particle number of 6 particles per sub-run of 0.1 mL, the Poisson distribution was applicable (compare Figs. 2i and 2j). This is similar to the result from a nominal concentration of 6 particles/mL with sub-run volumes of 1.0 mL (compare Figs. 2a and 2i).

Overall, the data show that the underlying distribution of the measurement results is only dependent on the particle number in the analyzed volume, but not on the particle concentration in the original sample material. For the combination of the low particle

concentration ranges and the low sample volume ranges, which are relevant for IVT products, measured particle numbers can be assumed to follow a Poisson distribution.

Theoretical Evaluation of the Ability to Distinguish Different Particle Concentrations

According to the evaluation of our experimental data, a Poisson distribution can be assumed as a model for the distribution of (small) particle numbers obtained from LO measurements. With the help of this model, the required analyzed volume to distinguish different particle concentrations was estimated.

As an example, Figures 3a-3d show Poisson distributions for mean particle numbers which are derived from the combination of a specified analyzed volume and 2 different particle concentrations: 2 particles/mL and 3 particles/mL. For 1 mL analyzed volume, the particle numbers are 2 and 3, respectively, and the distributions largely overlap (Fig. 3a). This means that with 1 mL analyzed volume, it is not possible to judge from a measurement result whether the true concentration in the sample was 2 particles/mL or 3 particles/mL.

To quantify the ability to distinguish different particle concentrations, a power analysis can be performed. The power is the probability that an effect (e.g., increase in particle concentration above a predefined limit) is observed if a specified significance level α is taken into account.²⁴⁻²⁶ Figure 3e shows the procedure for the example of a differentiation between 30 and 20 particles with a significance level of 5%: A critical value is defined in such a way that

Table 2
Influence of the Strategy of Data Collection/Processing on the Variability of the Final Particle Concentration Result

Collection/Processing of Data	Nominal Concentration 6 #/mL			Nominal Concentration 60 #/mL		
	Mean [# /mL]	Standard Deviation [# /mL]	RSD [%]	Mean [# /mL]	Standard Deviation [# /mL]	RSD [%]
Extrapolated particle concentration from individual sub-runs of 0.1 mL (200 sub-runs)	5.9	6.8	117	46.7	23.6	50
Particle concentration from individual sub-runs of 1.0 mL (200 and 198 sub-runs for 6 and 60 #/mL, respectively)	4.8	2.3	47	47.9	7.8	16
Sum of particle numbers in the 10 sub-runs of 0.1 mL which constitute 1 measurement (the same 20 measurements as in line 1)	5.9	2.9	49	42.8	8.3	19

Data from LO measurements of $25\text{-}\mu\text{m}$ polystyrene standard beads. For each data set, mean, standard deviation, and relative standard deviation (RSD) of the particle concentrations are listed (Particles $\geq 20 \mu\text{m}$ and $< 30 \mu\text{m}$).

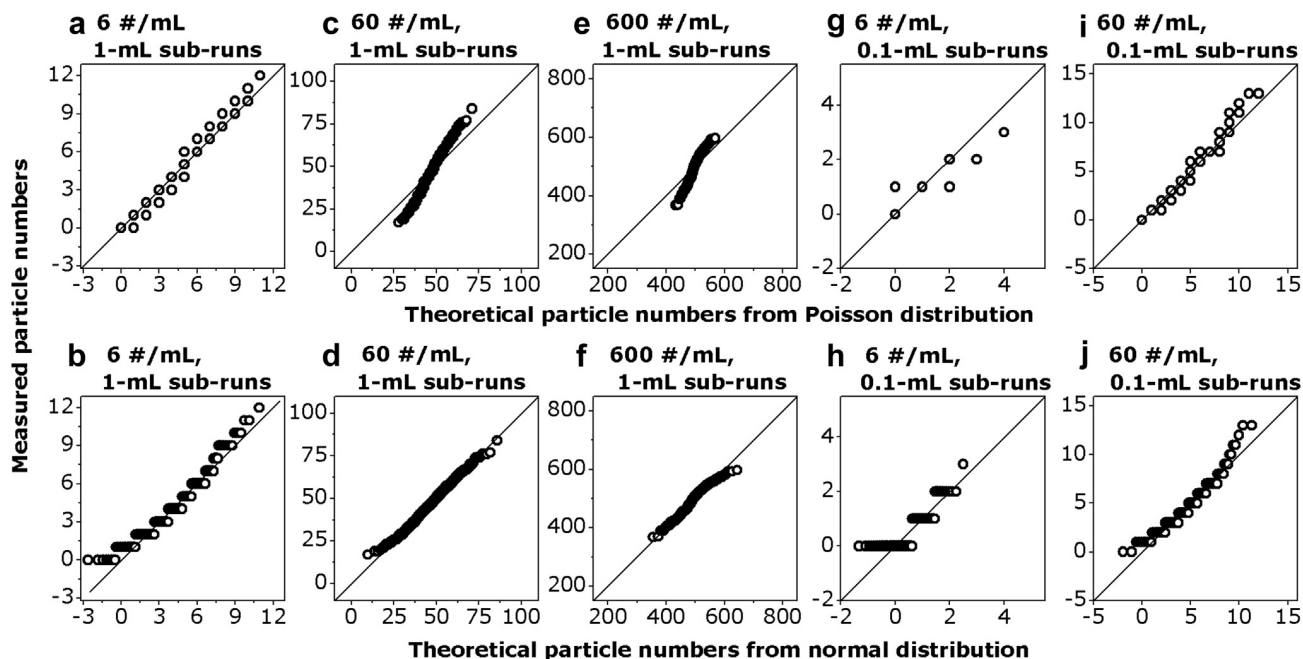


Figure 2. Quantile-quantile-plots for the comparison of experimental and theoretical distributions of particle numbers. Experimental data sets contain particle numbers obtained from the individual 1.0-mL (a-f) and 0.1-mL (g-j) sub-runs at the concentration level specified in the figure panels. Poisson distributions (a, c, e, g, i) were calculated based on the experimental mean of each data set. Normal distributions (b, d, f, h, j) were calculated based on the experimental means and standard deviations. Note: In (a), (g), and (i), several data points lie on top of each other owing to the restricted number of possible integer values in this low particle concentration range.

the probability is 5% that the measured particle number is above the critical value if the true particle number is 20. The power (probability of true-positives) is the probability $1-\beta$ that the measured value is above the critical value if the true particle number is 30.

The power is low for the differentiation of very close particle numbers (Fig. 3a). Furthermore, for 2 fixed particle concentrations, the power increases with increasing analyzed volume, because the gap between the mean expected particle numbers increases

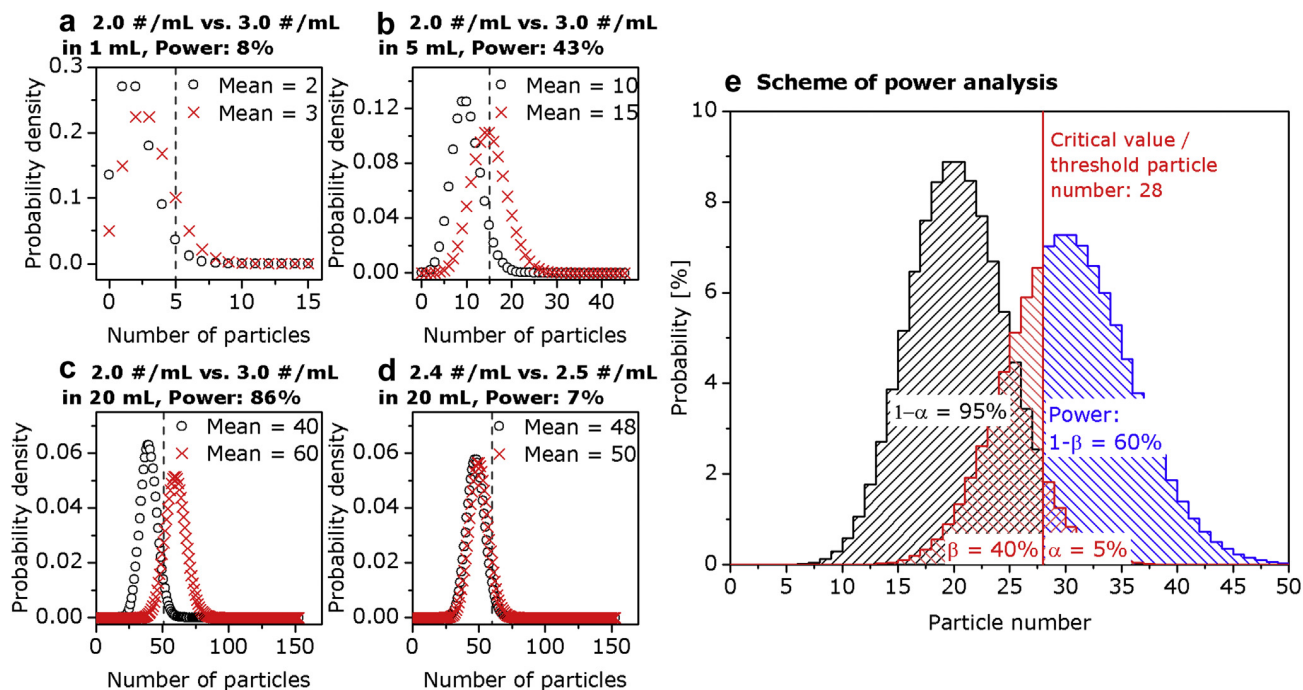


Figure 3. (a-d) Probability density of Poisson distributions with mean numbers of particles of (a) 2 and 3, (b) 10 and 15, (c) 40 and 60, and (d) 48 and 50. The dotted vertical lines show the particle number threshold (95% quantile of the Poisson distribution with the mean at the lower particle number) for a differentiation of the 2 particle numbers considered in each graph. (e) Scheme of the procedure for the power analysis based on the exemplary differentiation of 30 particles and 20 particles (corresponding to particle concentrations of 3 and 2 particles/mL in an analyzed volume of 10 mL).

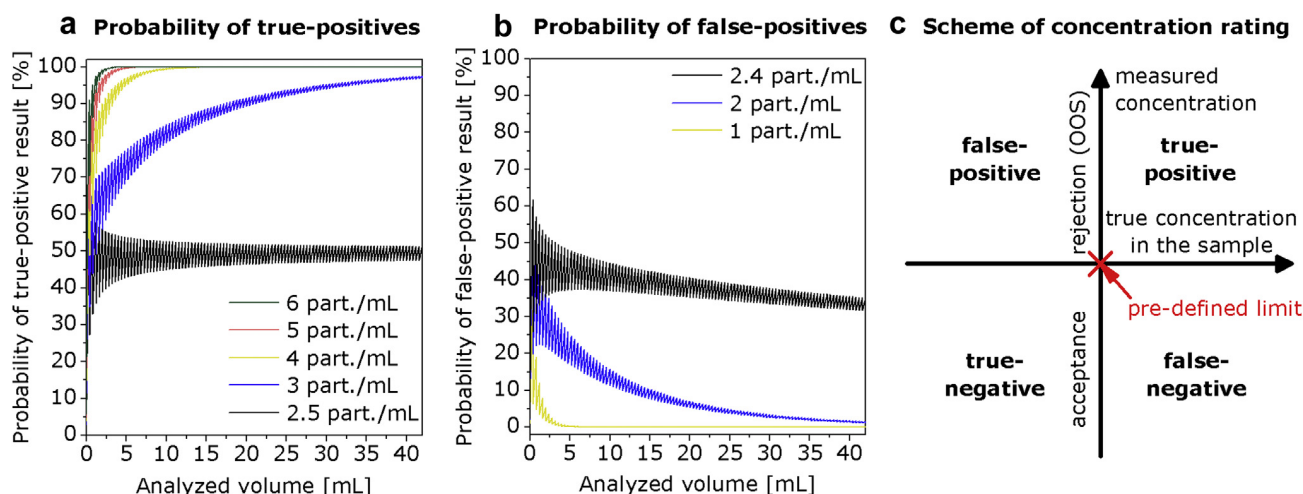


Figure 4. (a) Probability of true-positive results: Probability to encounter any number of particles above a predefined limit of 2 particles/mL in the analyzed volume for different true particle concentrations above the predefined limit. (b) Probability of false-positive results: Probability to encounter any number of particles above a predefined limit of 2 particles/mL at different true concentrations below the predefined limit. (c) Schematic representation of the rating of potential measurement results according to a predefined limit.

(compare Figs. 3a, 3b, and 3c). For a significant differentiation of a true particle concentration of 3.0 particles/mL from a true particle concentration of 2.0 particles/mL, more than 20 mL analyzed volume would be required (Fig. 3c).

In contrast to measurement results, which can only be integer particle numbers, real samples can have true particle concentrations between 2.0 particles/mL and 3.0 particles/mL. When aiming for a differentiation of true concentrations of 2.5 particles/mL (would be rounded up to 3 particles/mL, i.e., above the predefined limit) and 2.4 particles/mL (would be rounded down to 2 particles/mL, i.e., below the predefined limit), the abovementioned analyzed volume of 20 mL would only enable a power of 7% (Fig. 3d). This shows that the smaller the difference in particle concentration which is to be detected, the larger the required analyzed volume which can easily reach tens or hundreds of mL.

Probabilities of True-Positive and False-Positive Measurement Results

A different approach to estimate the required analyzed sample volume for statistically meaningful results is to evaluate the probabilities of true-positive and false-positive results (Fig. 4).

The probability of true-positive results increases with increasing distance of the true concentration to the concentration limit (Fig. 4a and supplementary information, Fig. S2). For example, for a predefined limit of 2 particles/mL and an analyzed volume of 5 mL, the probability of true-positives at a true particle concentration in the sample of 3 particles/mL (predefined limit + 1 particle/mL) would be 73%, whereas at a concentration of 4 particles/mL, the probability of true-positive results would be 96% (Fig. 4a). Furthermore, the probability of true-positive results increases with increasing analyzed volume; for example, for the predefined limit of 2 particles/mL and a true concentration of 3 particles/mL, the probability of true-positives increases from 73% with 5 mL to 90% with 20 mL (Fig. 4a).

For a true particle concentration in the sample which is directly at the predefined limit, the probabilities of both true-positive and false-positive results are approximately 50%, because the statistical distribution extends almost equally above and below the mean concentration. For example, for the predefined limit of 2 particles/mL, the probability of true-positives at a true concentration of 2.5 particles/mL (rounded up to 3 particles/mL, Fig. 4a) and the probability of

false-positives at a true concentration of 2.4 particles/mL (rounded down to 2 particles/mL, Fig. 4b) are approximately 50%.

For true particle concentrations below the predefined limit, the probability of false-positive results decreases with increasing distance of the true concentration to the predefined limit and with increasing analyzed volume (Fig. 4b and supplementary information, Fig. S3). For example, for the predefined limit of 2 particles/mL and a true concentration of 1 particle/mL, the probability of false-positives would be 5% already with an analyzed volume of 2 mL, whereas for a true concentration of 2 particles/mL, 23 mL would be required to obtain as low as 5% probability of false-positives (Fig. 4b).

In conclusion, a small analyzed volume seems to consume less sample when considering a single measurement, but the larger probability of false-positive results exceeding predefined limits might lead to more retesting, which increases the total required sample volume. Additionally, the risk to overlook particles which are present in the sample increases with decreasing analyzed sample volume as can be seen by the low probability of true-positives at small analyzed volumes.

Confidence When Rating Samples According to Predefined Limits

With predefined limits of 2–50 particles/mL, a high variability can compromise the significance of analysis results when small sample volumes, which contain only a few particles, are analyzed. Therefore, confidence intervals of hypothetical measurement results in this low particle concentration range were evaluated to assess the analyzed sample volume which is required to assure that a sample complies with a predefined limit. Confidence intervals provide the range of possible true particle concentrations in a sample which can be concluded from a measured particle concentration or number.

Figure 5 shows the maximum confidence levels at which the upper limit of the confidence interval for a hypothetically measured particle number (ordinates) does not exceed a predefined particle concentration limit (abscissas). For example, with a predefined concentration limit of 2 particles/mL, the allowed number of particles in an analyzed volume of 2 mL would be 4. However, already when 3 particles are measured in 2 mL, the confidence is only 56% that the actual particle number in the sample does not exceed 4 (Fig. 5a). Upper limits of confidence intervals at confidence levels of

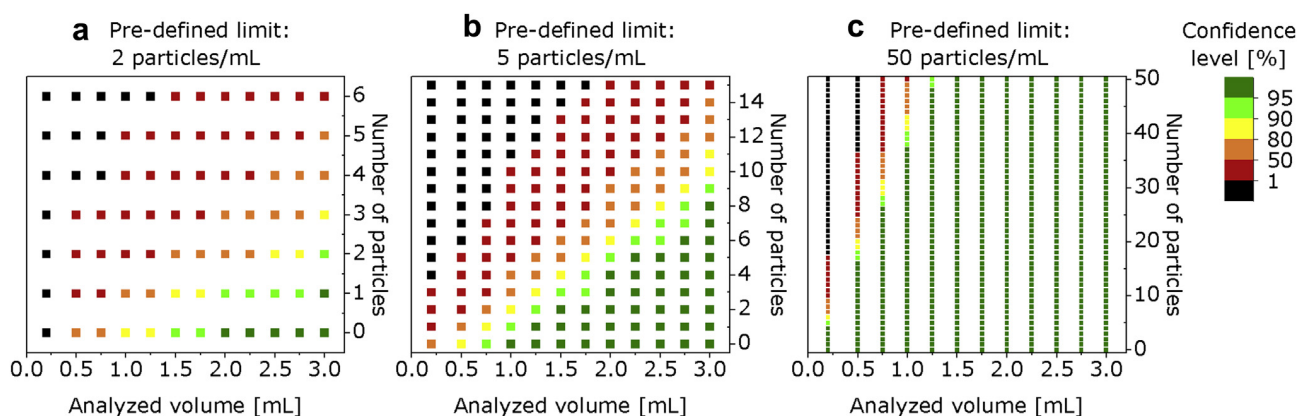


Figure 5. Confidence levels which would be achievable based on one-sided confidence intervals of a Poisson distribution when analyzing a defined sample volume, obtaining a defined particle number as measurement result, and aiming to state the compliance of the measured sample with a predefined concentration limit of (a) 2 particles/mL, (b) 5 particles/mL, and (c) 50 particles/mL. The black pixels in the graphs are those combinations of particle number and analyzed volume for which a confidence level cannot be determined, because the upper end of the 1% confidence interval would already exceed the particle number limit for the respective volume.

>56% would exceed the allowed number of 4 particles in 2 mL. Similarly, the data show that even if no particle is found, compliance with a predefined limit of 2 particles/mL can only be concluded with >90% confidence if a volume of at least 1.5 mL has been analyzed (Fig. 5a). To assure compliance with a predefined limit of 5 or 50 particles/mL, smaller sample volumes can be feasible (Figs. 5b and 5c).

The calculations above were carried out based on the assumption that a single measurement is performed. The confidence interval becomes smaller when replicate measurements are performed. For example, for a measurement result of 6 particles in 3 mL, the upper 95% confidence interval limit would be 12 particles (i.e., 4 particles/mL) with a single measurement (Eq. 1). When the same measurement result is obtained as the average of a triplicate measurement (3×3 mL), the upper limit of the 95% confidence interval would be 9 particles (i.e. 3 particles/mL) (Eq. 1). Thus, replicate measurements can increase the confidence in a measurement result while maintaining the same sample volume per individual measurement. However, the required total analyzed volume cannot be reduced by using replicates. This means that a strategy involving replicates cannot reduce the total consumption of sample material or the time required for sample pooling, but it can be helpful to reduce pooling risks by reducing the sample volume per pool.

Discussion

To judge the statistical relevance of measured particle counts, it is important to know the uncertainty of measurement results, which is determined, among others, by the underlying statistical distribution. In our study, we evaluated the distribution of subvisible particle concentrations measured by LO. We chose on purpose polystyrene standard beads for this systematic study, because they allowed the preparation of physically stable samples with well-defined nominal particle concentrations without the risk of changes in particle size or number during sample preparation and analysis, which would be expected for example for protein particles. Very recently, a new type of particle standard became commercially available (NIST reference material 8634), which is made from ethylene tetrafluoroethylene and mimics the optical properties of protein particles better than polystyrene particles. Future testing of the counting statistics with this new standard would be promising. However, the new standard was not yet commercialized and available in sufficient quantities at the time of the study discussed in this article.

In a previous study, a relative standard deviation of particle concentrations determined by LO of approximately 50% at 3 particles/mL has been reported.¹⁸ Our experiments showed a similar result with a standard deviation of approximately 2 particles/mL at mean measured particle concentrations of 4-5 particles/mL. This is a large variability compared with the particle concentration limits which are required for ophthalmic solutions by USP <789>. However, it is in accord with the assumption that for low analyte numbers, uncertainty is mainly determined by the statistical distribution of the analytes, namely Poisson distribution.²⁷

As the standard deviation of a Poisson distribution is proportional to the square root of the mean value of the distribution, the relative uncertainty induced by statistical analyte distribution decreases with increasing number of expected analytes in the analyzed volume and vice versa. As a result, uncertainty generated by statistical analyte distribution in the sample is small for high analyte numbers and is outweighed by experimentally induced uncertainty so that measurement results often follow a normal distribution. This was observed in our experiments starting from a nominal concentration of 60-600 particles/mL and an analyzed volume of 1.0 mL.

Owing to the increasing relative standard deviation with decreasing number of analytes, a minimum analyzed volume specific for concentration and desired precision exists, below which the uncertainty of the quantification becomes too large to provide a reliable result, even when homogeneity of the samples and high precision of the measurement instrument are assumed.^{19,27,28} Our theoretical calculations show that unfeasibly large analyzed sample volumes on the order of tens or hundreds of mL would be required to significantly distinguish similar particle concentrations, for example, 3 particles/mL and 2 particles/mL. Even with these large analyzed volumes, the probability is approximately 50% that a sample with a true particle concentration which is close to a predefined limit would be incorrectly rated as in or out of a given predefined limit.

For infinitely similar particle concentrations or an infinitely small difference between true particle concentration and predefined limit, differentiation is impossible. This raises questions concerning the interpretation of particle concentration limits and the rationale behind these limits. For example, USP <789> sets an upper particle concentration limit of 2 particles/mL $\geq 50 \mu\text{m}$. This can be understood in 2 ways: On the one hand, 2 particles/mL could be the upper limit above which a product might acquire unwanted properties. This would mean that a measurement result of 2 particles/mL with the measurement uncertainty discussed above would not provide sufficient certainty that the product is

within the acceptance criteria. Instead, a safety margin might be necessary when rating samples as below or above the limit. On the other hand, 2 particles/mL may have been set far below a potentially critical particle concentration, allowing true particle concentrations to be higher as long as the average measurement result does not exceed the limit. In this case, 2 particles/mL might represent a compromise for “as few as possible,” because 0 particles/mL is hard to measure in practice.

If usual measurement uncertainty of LO analyses is already included in the USP <789> limits, they may be understood as “measurement result limits,” allowing the true particle concentration in the sample to be higher. In this case, low analyzed sample volumes might be chosen as long as the standard deviation is on the same level that was assumed for the definition of the predefined limits. In contrast, if the predefined limits were to be understood as limits for the true particle concentration in a sample, measurement uncertainty would need to be considered when assessing the required analyzed sample volumes. In this case, the high analyzed volumes evaluated in the results section would be necessary, and safety margins between particle concentration limit and allowed measurement results might need to be considered.

The following 2 examples illustrate the levels of uncertainty obtained with different analyzed volumes which are relevant in terms of subvisible particle testing according to USP.

If USP <787> applies, an analyzed volume as low as 0.6 mL would be allowed. For a predefined limit of 2 particles/mL $\geq 50 \mu\text{m}$, 1 particle in 0.6 mL (1.7 particles/mL) would be below the predefined limit. Considering a 95% confidence interval, the average true particle number in an equivalent sample volume can be up to 4.7 particles if 1 particle is found (Eq. 1). For an analyzed volume of 0.6 mL, this means that the true particle concentration in a sample which passes the limit of 2 particles/mL can be up to 7.8 particles/mL. This exceeds the limit by almost 300%.

If USP <788> applies, the analyzed volume would be 15 mL. In this case and for the limit of 2 particles/mL, 37 particles is the largest accepted particle number (i.e., 2.47 particles/mL). The 95% confidence interval of 37 particles extends up to 48.7 particles (Eq. 1). As a result, a measurement result within the limit of 2 particles/mL with 15 mL analyzed volume can mean that the true particle concentration in the sample is up to 3.3 particles/mL, that is, exceeding the limit by approximately 50%. Although an increase in analyzed volume from 0.6 mL (USP <787>) to 15 mL (USP <788>) increases the reliability of a measurement result (i.e., an appropriate confidence interval), the example above shows that the limit of 2 particles/mL, as specified in USP <789>, might not be meaningful when judging on the acceptability of a sample. Consequently, the meaning of the currently provided limits of USP <789> should be discussed.

In conclusion, both statistical and experimental restrictions need to be considered when setting up a pooling and measurement procedure. Experimentally, the analyzed sample volume may be restricted, for example, by the sample unit volume or the risk of particle intake during pooling. A minimum analyzed volume which provides precise and reliable results can be defined by considering confidence intervals and probabilities of true-positive and false-positive results. For each combination of particle concentration limit and accepted rate of false-positive results (which might lead to re-testing), a minimum analyzed sample volume exists, below which even a measurement result of 0 particles does not provide reasonable certainty.

Conclusions

The relative standard deviation of particle concentrations obtained from LO measurements is generally large, and it increases with decreasing particle concentration. Particle counts in the low

concentration range which is relevant for USP <789> are Poisson distributed.

Our theoretical evaluations based on these findings showed that large sample volumes on the order of tens or hundreds of mL would be required to significantly distinguish similar particle concentrations (e.g., 3 particles/mL from 2 particles/mL). Similarly, testing of large volumes would be required to correctly classify a sample as below or above a predefined limit. The lower the analyzed sample volume, the larger the concentration range within which differentiation is not possible. Finally, below a minimum analyzed volume (specific for each predefined limit), even a measurement result of 0 particles is not meaningful.

Nevertheless, for IVT products and many other biopharmaceuticals, large analyzed volumes would require extensive pooling which increases the risk of external particle intake and masking of vial-to-vial variability, as well as the consumption of material and time. Therefore, we suggest to define an analyzed volume that provides reasonable certainty dependent on the container type and fill volume of the drug product. For that, statistical parameters, such as probabilities of true-positives and false-positives and confidence intervals, should be evaluated based on previous knowledge on the specific product, for example, expected particle concentrations and product variability. The counting statistics and required analyzed volumes discussed here, apply not only to the example of LO for ophthalmic products but also generally to analyses which aim for the quantification of analyte species which occur in similarly small discrete numbers in the analyzed sample volume.

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