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Particle size by design: Standardizing measurements for complex topical drug product assessment

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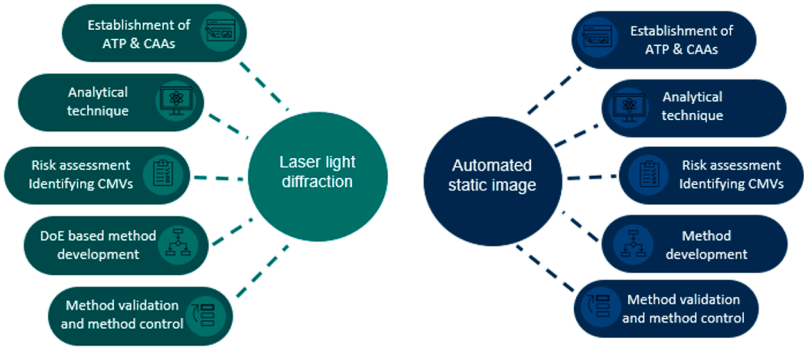
HIGHLIGHTS

- First time AQbD approach applied to analyze particle size via automated microscopy and laser light diffraction.
- Optimization of laser light diffraction method using face-centered central composite design (FcCCD).
- Precise particle size measurements were obtained from both automated microscopy and laser light diffraction techniques.
- Image analysis produces morphology data that pure laser diffraction may not.
- A combined laser diffraction-image analysis method is ideal for analyzing complex semisolid formulation.

GRAPHICAL ABSTRACT



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ABSTRACT

The physicochemical and biopharmaceutical properties of drug substances and dosage forms can be significantly influenced by particle size. However, the diversity of equivalent particle diameters generated by different methods poses a fundamental challenge in particle size analysis. This study aimed to develop an Analytical Quality by Design (AQbD) approach to accurately assess the particle size of a complex formulation – clobetasol propionate (CP) 0.5 mg/g cream – through automated microscopy (AM) and laser light diffraction (LD). Additionally, Raman spectroscopy was utilized to determine the chemical composition of the formulation particles. In the AQbD approach, prior knowledge was considered for the construction of the Ishikawa diagram and estimate failure mode and effects analysis (FMEA). The methods were developed following the ICH Q8-Q10, and ICH Q14 guidelines, and validated according to ICH Q2, ISO 13320:2020, and EP2.9.31./USP<429>. Results indicate that a trade-off between the techniques must be established for a particle size by design: while LD offers higher

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throughput and more precise values at the expense of peak resolution and broadening, AM has higher variability but more reliable information in terms of size and shape analysis. The validated methods successfully demonstrated the implementation of an AQbD method in the definition of particle size methods.

1. Introduction

The primary goal of the research and development (R&D) process in pharmaceutical industry for a new pharmaceutical formulation is to design a high-quality product, with a reliable manufacturing process, that consistently delivers the intended performance. To achieve so, formulation studies should involve a comprehensive scientific understanding of the physicochemical characteristics of both active pharmaceutical ingredient (API) and excipients. This knowledge is vital to guarantee the product pharmacological targets defined by the API molecules, alongside with the product specifications, and suitable manufacturing controls [1,2]. In what concerns topical drug products, especially semisolid dosage forms, a detailed understanding of the product microstructure is also deemed necessary, as changes in these attributes caused by raw materials, stability issues or manufacturing processes, may have a direct repercussion in product performance, which may ultimately affect product efficacy. Within this scope, when designing a topical generic product, microstructure equivalence should be supported. Regulatory agencies, including both the European Medicines Agency (EMA) and the Food and Drug Administration (FDA), emphasize the importance of employing specific methods in this regard [3]. Noteworthy guidelines include the EMA's quality and equivalence of topical guideline [4], the FDA's Physicochemical and Structure (Q3) Characterization of Topical Drug Products submitted in ANDAs [5], and the United States Pharmacopeia (USP-NF) general chapter of topical and transdermal drug products [6].

It is important to note that according to semisolid nomenclature, different microstructure complexities can be denoted. As such, in monophasic systems (e.g. ointments, gels), there is a simple microstructure; however, the scenario changes in multiphasic systems (e.g. creams, emulsions) where product microstructure is far more complex. According to regulatory requirements, during the development of a topical generic product, after the documentation of the qualitative and quantitative equivalence, the microstructure equivalence should be documented [7–11].

The characterization of structural organization of matter includes the determination of the particle size by means of appropriate techniques, such as manual microscopy (MM) or automated microscopy (AM), morphologically directed Raman spectroscopy (MDRS®), dynamic light scattering (DLS), laser light diffraction (LD), sieve analysis, and voltmeter [5,12]. Note that the term "particle" in this work encompasses solid particles and/or droplets, considering all types within this context. These techniques are essential during the R&D stage to understand the relationship between particle characteristics, and the overall physicochemical and sensory properties of semisolid dosage forms, including stability, texture, appearance, and efficacy. If a single technique cannot accurately determine the complete particle size distribution, the use of a second technique may be necessary [13,14]. Generally, the width of the distribution curve is of utmost importance. Percentiles (D-values) are commonly used in particle size measurements, representing specific points in the cumulative distribution curves (volume or number). These values should be followed by a number, for example, D_x. The three most frequently used D-values for volume distribution are Dv_{10}, Dv_{50} and Dv_{90}. The Dv_{50}, or median, regards the maximum particle diameter below which 50 % of the sample volume exists. Similarly, 90 % of the population lies below the Dv_{90} point, while 10 percent lies below the Dv_{10}. Note that "v" denote volume distribution [15–20].

Automated microscopy (AM), also known as static image or static optical microscopy analysis, offers several advantages. It enables rapid measurement of thousands of particles and provides information on both their size and shape descriptor distributions. This approach proves to be especially beneficial for assessing narrow size distributions and characterizing diverse sample types, including pharmaceuticals, such as API, excipient, product under development and final product. By generation of high-resolution images, automated microscopy provides precise size and shape information, making it a valuable technique, especially for research and development purposes [14].

On the other hand, laser light diffraction analysis is particularly well-suited for routine measurements of bulk products, powders, granules, and suspensions. This method is known for its high sample throughput, reliability, and excellent reproducibility. In laser light diffraction measurement, a laser beam is directed through a dispersed particle sample, and the angular variation in the intensity of the scattered light is measured. This data is then analyzed to calculate the size of the particles responsible for the scattering pattern [14]. The theoretical scattering pattern of a single spherical homogeneous particle is given by Mie theory in general. If the particle has a size relatively large and/or is opaque, Fraunhofer diffraction theory is also available and delivers equivalent results without the need of knowing particle's optical properties [21,22].

In automated microscopy analysis, a wide range of morphological parameters can be obtained and quantitatively evaluated for all identified particles. The particle size distribution can be determined based on various size definitions, including particle width, particle length or diameter of the equal area circle. Additionally, automated microscopy analysis enables particle shape analysis, allowing for the characterization of parameters such as aspect ratio, diameter, circularity, convexity, elongation, intensity, and solidity [14].

Due to the importance of particle size determination, together with the complexities inherent to these methods and the sample matrix from which this parameter is retrieved, there is a permanent need to establish well-defined and robust methodologies fit for this purpose. Analytical Quality by Design (AQbD), introduced in 2018, refers the application of the Quality by Design (QbD) concept, strongly recommended by the FDA and EMA, towards the development of analytical methods. AQbD aims to optimize all stages of the analytical procedure life cycle by identifying and controlling critical method variables (CMVs) and risk factors throughout the entire analytical protocol. In conjunction with this, the use of Design of Experiments (DoE) enables gaining a comprehensive understanding of how CMVs influence the critical analytical attributes (CAAs) [23,24].

In the present study, two methods for evaluating particle size were conducted and compared. As a case study, a Clobetasol propionate (CP) 0.525 mg/g cream formulation was used [25–27]. Considering this oil-in-water emulsion as a complex formulation, particle size distribution plays a pivotal role in product quality. Studies on emulsions have indicated that the type of emulsion significantly affects skin drug delivery. Due to the evaporation of the volatile components of the emulsions, there is a possibility of resizing the droplets [28–30]. Additionally, some studies have shown that the skin penetration depends on the size of the droplets in the emulsion, indicating that smaller droplet sizes increase the probability of skin absorption of drug molecules under the same conditions [31–33]. Therefore, controlling the particle size of the cream formulation, within a robust analytical method, is crucial to enhancing product quality and performance.

A comprehensive AQbD study was conducted for laser light diffraction analysis, while a partial AQbD approach was applied to automated microscopy analysis in accordance with the ICH Q14 guideline and ISO 13322–1:2014. Subsequently, both methods were successfully validated following of ICH Q2(R2) guideline, USP chapter <429>, EP chapter

Table 1
Analytical Target Profile (ATP) established for the laser light diffraction analysis of CP cream.

ATP		Target	Justification	Specification
Product/Sample		Determine the particle/droplet size of an o/w clobetasol propionate cream formulation	Development and validation of a laser light diffraction particle size method to characterize the microstructure of a complex formulation. The cream placement must be adequate to ensure a representative sample with suitable volume for the particle size measurement. Sample splitting techniques such as rotating riffler or the cone and quartering method may be applied.	N.A.
Analytical technique		Laser light diffraction particle size analysis	Measurement of the particle size distribution. This measure can be carried out using dry or wet modes. Wet mode specifically applies to semisolid samples.	N.A.
Instrument		Laser light diffraction particle size analyzer equipped with sequential combination of measurements with red and blue light sources to measure across the entire particle size range	The angular scattering intensity data is analyzed to calculate the size of the particles responsible for creating the scattering pattern, using Mie theory or Fraunhofer diffraction of light scattering. The particle size is reported as the volume-equivalent sphere diameter.	N.A.
Application		Particle size	Method should be able to determine the distribution values in a complex cream formulation for product development or routine analysis. The assessment of the particle size is indispensable within Topical Generic product (TGP) development in order to provide comparative microstructure data to assess the feasibility of manufacturing process, as well as the effectiveness of formulation.	N.A.
CAAs		Dv_{10}, Dv_{50} and Dv_{90}	Laser light diffraction particle size allows for a robust and reliable determination of the distribution values. These should meet their formal and commonly accepted quality criteria.	Dv_{10} RSD $\leq$ 15 %; Dv_{50} RSD $\leq$ 10 %, Dv_{90} RSD $\leq$ 15 % (USP <429> [38])
Method validation	Precision (Repeatability and Intermediate precision)	Closeness of agreement between independent test/measurement results obtained under established conditions	This parameter includes variability measurement stemming from sampling and dispersion	Dv_{10} RSD $\leq$ 15 %; Dv_{50} RSD $\leq$ 10 %, Dv_{90} RSD $\leq$ 15 % (USP <429> [38])

Key: ATP: analytical target profile. CP: clobetasol propionate; CAAs: critical analytical attributes; Dv: distribution values; NA: not applicable.

(2.9.31.), and ISO 13320:2020. To our knowledge, this is the first report addressing the application of an AQbD strategy in the development of a standardized analytical method targeting semisolid particle size determination, followed by formal validation studies.

Taking into account the current regulatory background, the specific objective of this work is to propose a workflow, based on AQbD principles, towards the development and validation of the LD and AM methods applied to semisolid formulations.

2. Materials and methods

2.1. Materials

Clobetasol propionate, chlorocresol, glyceryl stearate, cetostearyl alcohol, citric acid, sodium citrate, propylene glycol, beeswax, and purified water were supplied by Laboratórios Basi Indústria Farmacêutica S.A. (Mortágua, Portugal).

2.2. Preparation of clobetasol propionate cream formulations

Clobetasol propionate o/w cream formulations were prepared using a conventional method with an Ultra-Turrax X 10/25 equipment from Ystral GmbH (Dottingen, Germany). The continuous and dispersed phases of the cream were prepared separately and heated to 60 °C. Subsequently, the active pharmaceutical ingredient was dissolved in the dispersed phase. The resulting cream formulations were stored at 20–25 °C. Batches of 0.5 kg were considered. Note that these formulations were utilized for method development and validation purposes. Furthermore, commercial formulations of CP were applied to assess the applicability of the method.

2.3. Cream pre-treatment for laser light diffraction

Samples for LD analysis were previously diluted by adding 0.5 g of CP cream formulation into 50 mL of deionized water and homogenizing for 30 minutes using a magnetic stirring plate. Mastersizer 3000 instrument (Malvern Panalytical, UK) was utilized, equipped with hydro MV and Aero S accessories. The detector employed a log-spaced array, while the optics included red and blue light sources. Water (refractive index: 1.33) was used as the dispersant, Mie scattering theory, and the obscuration level between 10 – 20 % for sample measurement were considered. The particle refractive index for the CP cream was set to 1.56, while the absorption index was set to 0.01 [34–36]. All measurements were performed in triplicate, and the mean particle size values of Dv_{10}, Dv_{50} and Dv_{90} were calculated. Statistical modeling was performed by JMP v.17 software (Cary, IL, USA).

2.4. Cream pre-treatment for automated microscopy

To improve the analysis quality, morphological filtering techniques were applied to the raw image data in order to eliminate outliers. For the analysis process, small amounts of cream samples were smeared in a glass slide, ensuring a thin and even distribution.

Particle analysis was conducted using Morphology 4 instrument (Malvern Panalytical, UK) equipped with various lighting models, including brightfield, diascopic and episcopic LED lighting, as well as darkfield and episcopic lighting, unified with an 18 MP detector camera and Nikon CFI 60 optical system. Particle imaging analysis was performed using a 50x magnification lens, which allowed for the detection and analysis within the size range of 0.5–50 μm. The data obtained were analyzed using the Morphologi v.4.82.0002 software (Malvern, UK). For chemical identification, Morphologically Directed Raman Spectroscopy, (MDRS®), Morphology 4-ID instrument (Malvern Panalytical, UK) was

Table 2
Analytical Target Profile (ATP) established for the automated microscopy analysis of CP cream.

ATP		Target	Justification	Specification
Product/Sample		Determine the particle/droplet size of an o/w clobetasol propionate cream formulation	Development and validation of an automated microscopy method for the pharmaceutical formulation. As only a small amount of material is needed to prepare a test sample, this should be subdivided in a manner that ensures that the test sample is representative of the whole.	N.A.
Analytical technique		Automated microscopy	Measurement of the particle shape parameters: aspect ratio, circle equivalent diameter, convexity, elongation, intensity, solidity, and high sensitivity circularity	N.A.
Instrument		Automated microscopy to measure across the entire particle size range	Automated microscopy offers the operator use without variability and greatly improving statistical robustness	N.A.
Application		Particle size	Method should be able to determine the distribution values in a glass slide of cream formulation for product development or routine analysis. Measuring the size and shape of particles provides indispensable qualitative data to assess the feasibility of the manufacturing process or the final effectiveness of formulation.	N.A.
CAAs		Dv_{10}, Dv_{50} and Dv_{90}	Automated microscopy particle size allows a robust and reliable determination of the distribution values. Should meet their formal and commonly accepted quality criteria.	Dv_{10} RSD $\leq$ 15 %; Dv_{50} RSD $\leq$ 10 %, Dv_{90} RSD $\leq$ 15 % (USP <429> [38])
Method validation	Precision (Repeatability and intermediate precision)	Closeness of agreement between independent test/measurement results obtained under stipulated conditions	This type of repeatability includes variability due to sampling and dispersion	Dv_{10} RSD $\leq$ 15 %; Dv_{50} RSD $\leq$ 10 %, Dv_{90} RSD $\leq$ 15 % (USP <429> [38])

Key: ATP: analytical target profile. CP: clobetasol propionate; CAAs: critical analytical attributes; Dv: values; NA: not applicable.

Table 3
Failure mode and effect analysis criteria to setup analysis scores.

	Score	Criteria
Severity (S)	1 (very low)	No impact on method quality
	2 (low)	No impact on method quality
	3 (average)	Noticeable impact to method quality, but can be recovered by reprocessing
	4 (high)	Definite impact to method quality that may require attention
	5 (very high)	Very severe effect, require particular attention
Occurrence (O)	1 (unlikely)	Negligible risk which does not require attention
	2 (remote)	Failure only seen once or twice
	3 (occasional)	Failure potential has been noted
	4 (moderate)	Moderate probability occurrence
	5 (likely)	Highly severe effect which require utmost attention
Detection (D)	1 (very low)	Easily detectable; negligible risk which do not require attention
	2 (low)	Good detectability: possess minor risk which can be corrected
	3 (average)	Detectable; risk which can be corrected
	4 (high)	Not easily detectable; risk require attention
	5 (very high)	Very difficult detectable; risk which require immediate attention

Note: adapted from Chiarentin et al. [9].

used, along with the knowltAll® software. MDRS® measurements were conducted using a 50x objective to obtain the spectrum itself.

2.5. AQbD particle size method development

2.5.1. Analytical target profile

The analytical target profile (ATP) outlines the desired performance objectives of a method and specifies the performance criteria to be employed during the measurements. A proper utilization of the ATP guarantees that the developed method is suitable for its intended purpose [37]. In Table 1 and Table 2, the ATP elements are presented, encompassing the optimization and validation of both particle size techniques. Considering their significance in both techniques, the distribution values (Dv_{10}, Dv_{50} and Dv_{90}) of the sample were identified as critical analytical attributes (CAAs).

2.5.2. Risk-management methodology

A risk-based approach should be employed to identify procedures within a control strategy that may pose moderate to high risks and therefore warrant the application of AQbD principles. Risk assessment involves identifying parameters that could have a negative impact on CAAs. These assessments help in recognizing inherent analysis risks and developing measures, processes, and controls to mitigate these risks. Ishikawa diagram was utilized to identify the potential sources of risk control. Failure mode and effect analysis (FMEA) were also employed to assess and select the critical failure modes that required further investigation. Once the failure modes were identified, risk reduction strategies were implemented to eliminate, contain, or control potential failures. The risk priority number (RPN), given by RPN = Severity (S) x Occurrence (O) x Detectability (D), was used as an indicator. An RPN value exceeding 30 was considered to indicate a high-risk failure mode. The criteria used for determining the RPN are presented in Table 3. FMEA systematically breaks down the analysis of complex processes into manageable steps, which enables the establishment of a more focused risk mitigation plan [2].

2.5.3. Method development and optimization

2.5.3.1. Laser light diffraction analysis. After conducting a risk assessment, it is advisable to use design of experiments (DoE) for method development to screen and/or optimize the method conditions. A response surface methodology (RSM) approach was employed to determine the optimal analysis conditions during method development. Face-centered central composite design (FcCCD) method was applied for laser light diffraction experimental conditions. The FcCCD method involves a second-order model that combines a two-level factorial design (2^n) or fractional factorial design (2^{n-k}), one central point and 2k outer points, denoted as axial or star points. In the case of an FcCCD, with two factors, both factorial and star points present the same negative and positive distance from the central point [39,40]. The selection of variables is crucial as it influences the experimental results, as well as their interpretation.

The coefficients for the second-order polynomial model were estimated using least squares regression. The proposed models for each CAA were described by the quadratic polynomial function in Eq. (1):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_{11}^2 + \beta_{22} X_{22}^2 \quad (1)$$

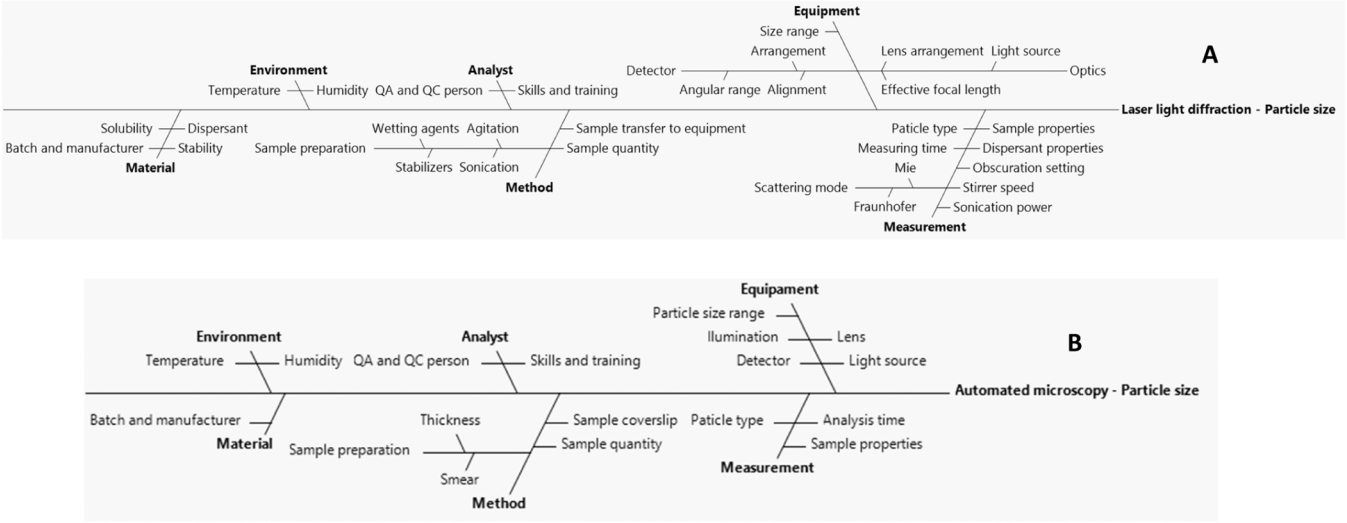

Fig. 1. Ishikawa diagram depicting cause-and-effect relationship on the potential CAAs of laser light diffraction (A) and automated microscopy (B) analytical methods. Key: CAAs: critical analytical attribute; QC: quality control; QA: quality assurance.

where Y represents the evaluated response associated with each factor level combination, β_0 is the response in the absence of effects, β_1 and β_2 are the linear coefficients of the factors X_1 and X_2, respectively, β_{12} is the interaction coefficient between X_1 and X_2, and β_{11} and β_{22} are the quadratic terms that allow the prediction of the curvature of the model. Non-significant terms were removed by backward selection, in which higher *p*-values terms are sequentially removed (*p*-value $>$ 0.05 threshold).

The selection of variables is crucial as it influences the experimental results, as well as their interpretation. To assess the statistical significance of the experimental parameters in the regression model, both Student *t*-test and ANOVA were conducted.

2.5.3.2. Automated microscopy analysis. The essential aspects of performing manual optical microscopy also apply for automated microscopy. Specifically, sample preparation is of paramount importance, followed by generating appropriate contrast. Typically, a thin plane is created by placing the sample on a microscope glass slide, and then cover it with a coverslip lined with a low vapor pressure fluid, such as mineral oil, that should be selected upon the sample type [16]. To acquire accurate size measurements, a uniform illumination across the entire field of view should be registered, alongside with a contrast-enhancing illumination method. The selection of the magnification should provide an adequate number of pixels for the smallest particle while maintaining the desired measurement accuracy. Additionally, the image focus should be set to achieve optimal particle sharpness. The number of pixels in smallest dimension of a particle is also relevant when measuring linear dimensions or combinations. For initial cream samples, the largest possible scan area was selected to maximize the number of imaged particles and ensure an ample particle count for an adequate description of the sample distributions [41]. The sample was observed manually to determine the optimum magnification for the measurement.

2.5.3.2.1. Chemical identification. Regarding Morphological-Directed Raman Spectroscopy (MDRS®) on the Morphologi 4-ID, the API and excipients were analyzed and identified separately using this technique. They were added to the library as individual components. Subsequently, the final product was analyzed to confirm that all API particles were dissolved during the manufacturing process. Additionally, a quantity of the API was intentionally added to the final product to differentiate between the API particles and cream droplets.

For Raman spectroscopy, the background signal was subtracted from the blank zone of the glass slide. The correlation values with the library components are listed at the bottom of the overlay, arranged in the order of the best match. The "best correlation" option serves as a quick and straightforward method for selecting and classifying a particle against multiple reference spectra.

2.6. Method validation

Typically, the validation procedure for a method involves assessing its specificity, linearity, range, accuracy, precision, and robustness. However, in particle size analysis using LD and AM, only the precision parameter, was determined [22].

The achievable precision of the method primarily depends on the characteristics of the material being analyzed (e.g., whether it is milled or not, its robustness/fragility, the width of its size distribution, etc.). The required precision, on the other hand, depends on the specific purpose of the measurement. It is not possible to establish mandatory limits for precision as per the USP <429> chapter and the EP chapter (2.9.31.), as the acceptable levels may vary significantly from one substance to another [38,42–44].

The precision of an analytical method procedure reflects the agreement between a series of measurements obtained from multiple samplings of the homogeneous sample under specified conditions. In the case of laser light diffraction and automated microscopy measurements, the precision evaluation was conducted through assessments of repeatability and intermediate precision involving another analyst, expressed as the percentage relative standard deviation (% RSD). The acceptance criteria for precision were set as % RSD $\leq$ 10 % for the central value of the distribution (Dv_{50}). For the values at the sides of the distribution (Dv_{10} and Dv_{90}), the acceptance criteria were slightly less stringent, such as $\leq$ 15 % [38]. However, for particles below 10 μm in size, these values should be doubled. Six replicates were considered and for each replicate 3 measurements were performed, in order to ensure statistical reliability.

3. Results and discussion

3.1. AQbD particle size method development

3.1.1. Risk-management methodology

Quality risk management was assessed through a risk assessment approach to identify and prioritize parameters that could impact method performance and compliance with the ATP [45]. The risk analysis for

Table 4
Failure mode and effect analysis for establishing the CMVs for LD analysis.

Failure Mode	Failure Effect	Severity (S)	Cause(s) of Failure	Occurrence (O)	Process Control	Detection (D)	RPN* score	Recommended action(s)
Material: dispersant	Dispersant application has to be appropriate	4	Sample dissolution; non-uniformed; inappropriate viscosity; opaque; same refractive index to the sample; chemical incompatibility with the instrument	3	Check the literature for information on dispersant properties	2	24	Choose an appropriate dispersant.
Method: sample preparation	Sample pre-treatment has to be appropriate	4	Amount of sample	2	Check the method developed	3	24	Selection of the liquid; Wetting the sample as it is placed in the liquid; Adding energy to de-agglomerate the particles; Stabilizing the mixture using appropriate chemical agent; and appropriate agitation, and sonication.
Equipment: detector	Equipment does not work	2	Thermal difference: results in "beam steering", where a larger signal reaches the inner or low angle detectors	1	Equipment qualification/ calibration	1	2	Qualification and/or calibration of the system.
Equipment: optics	Equipment does not work	1	Optical model artifact peaks: are associated with the complex refractive index	1	Equipment qualification/ calibration	1	1	Qualification and/or calibration of the system. Change the red or blue lights.
Measurement: scattering mode	Wrong measurement	2	Inappropriate scattering mode	3	Check the method measurement	1	6	Selection of an appropriate mode Mie theory or Fraunhofer approximation.
Measurement: stirrer speed	Wrong stirrer speed	5	Low speed, larger particles could be setting, and fine particles are being sampled disproportionately	4	Check the method measurement	3	60	Choose a suitable stirrer speed for each type of sample
Measurement: sonication power	Wrong sonication power	5	Sonication during the analysis can produce differences in particle size. Excessive ultrasonic energy can cause primary particulate to fracture in some cases.	5	Check the method measurement	3	75	Choose a suitable sonication power before starting the analysis
Measurement: obscuration setting	Amount of sample	3	Inadequate range of obscuration	3	Check sample concentration	3	27	To determine appropriate obscuration.

Key: RPN: Risk Priority Number (RPN = severity (S) x occurrence (O) x detection (D)); CMVs: critical method variables; LD: laser light diffraction.

Table 5
Failure mode and effect analysis for establishing the CMVs for automated microscopy measurements.

Failure mode	Failure Effect	Severity (S)	Cause(s) of Failure	Occurrence (O)	Process Control	Detection (D)	RPN* score	Recommended action(s)
Analyst: skills and training	Equipment use	4	Equipment operations, and uncertain results	3	Review stander operational procedure (SOP)	2	24	Analyst should be trained.
Method: sample preparation	Sample pre-treatment has to be appropriated	5	Unable to measure and provide data on particle size, shape, and transparency	3	Check the method developed	3	45	The glass slide should be prepared with care and before the microscopy analysis to avoid the sample drying.
Equipment: particle size range	Inappropriate measurement	2	The image of the particle size could not be clear	1	Review standard operational procedure (SOP)	1	2	Equipment could be able to analyze a large particle size range according to appropriate lens.
Equipment: lens	Inappropriate measurement	4	Dust, fingerprints, or other debris on the lens	2	Review standard operational procedure (SOP)	1	8	Regular cleaning with appropriate cleaning materials.
Equipment: illumination	Inappropriate measurement	4	Poor image quality	3	Review standard operational procedure (SOP)	1	12	Choose a suitable illumination. White light: brightfield, diascopic or episcopic

Key: RPN: Risk Priority Number (RPN = severity (S) x occurrence (O) x detection (D)); CMVs: critical method variables.

both particle size techniques was performed in two stages, the first one regarded an Ishikawa diagram for establishing a whole picture risk scenario, while the second one considered a FMEA tool for a fine-tuned analysis.

3.1.1.1. Identifying potential CMVs. In the Ishikawa diagram (depicted in Fig. 1), the potential causes of non-compliance were identified, thus enabling the identification of the CMVs that have the highest likelihood of leading to failures. In what concerns the laser light diffraction

Table 6
Experimental factors and levels used in the FcCCD for LD analysis.

	Levels		
Process parameters (CMVs)	**−1**	**0**	**+1**
Sonication power (%)	0	50	100
Stirrer speed (rpm)	500	1750	3000
Responses (CAAs)	$Y_1 = Dv_{10}$		
	$Y_2 = Dv_{50}$		
	$Y_3 = Dv_{90}$		

Key: FcCCD: Face-centered central composite design; LD: laser light diffraction; CMVs: critical method variables; CAAs: critical analytical attributes.

Table 7
Design matrix used for optimization of LD analysis.

Experiment	Code	Sonication power (%)	Stirrer speed (rpm)
1	- -	0	500
2	- +	0	3000
3	+ -	100	500
4	+ +	100	3000
5	a0	0	1750
6	A0	100	1750
7	0a	50	500
8	0 A	50	3000
9	00	50	1750

Key: LD: laser light diffraction.

technique, factors such as humidity, temperature, sample transfer to equipment, sample quantity and stability were considered as noise factors. Other parameters, such as the stirrer speed and sonication power, were also treated as control factors. On the other hand, for the automated microscopy analysis, factors such as environment, analyst, equipment, material, method, and measurement were classified as experimental factors. Each category provided valuable insights into factors that can influence the CAAs related to particle size and affect the method performance [46,47].

3.1.1.2. Ranking CMVs. In the second stage, a FMEA approach was utilized to rank variables using RPN. The CMVs were identified based on their final scores, as presented in Table 4 and Table 5. All factors identified in the Ishikawa diagram were evaluated regarding their severity, occurrence, and detectability. Regarding LD methodology, stirrer speed and sonication power were the method parameters with the highest RPN score, and those parameters will be applied for DoE investigation. In addition, a response surface methodology was specifically employed for laser light diffraction analysis to develop a robust method. This involved the creation of a mathematical model that describes the relationship between the CMVs and method performance. Through optimization of the CMVs within the defined range, the method can be enhanced in terms of robustness and reliability.

Regarding AM methodology, sample preparation was the method parameter with highest RPN score as shown in Table 5. The remaining failure modes, with ranging severity, occurrence, and detectability, were considered to be non-critical and considered easily resolved by the corresponding mitigation strategy [48].

3.2. Optimization of the particle size analysis method

3.2.1. Laser light diffraction analysis

The laser light diffraction analysis is based on the angular distribution of scattered light intensity by particle size [21]. The type of particle being analyzed should be specified, by distinguishing between non-spherical and spherical particles. Non-spherical particles are suitable for irregularly shaped or rough surfaced particles such as milled or crushed materials. On the other hand, spherical particle analysis is applicable for perfectly spherical particles, such as polymer latex samples or emulsion droplets. The main limitations and particularities of this method will be briefly addressed in what follows [49].

For scattering matrix calculations, which are mainly based on the signal at each detector element, per unit volume of particles in specific size classes, the application of either Mie theory or the Fraunhofer approximation is common. The choice of the approach to follow is dependent upon the size range of the particles in the sample being analyzed. When using Mie theory, the refractive indices of the particles and medium, or their ratio, should be set to calculate the model matrix [21,50]. The latter provides the possibility of obtaining precise size distributions for particles of all sizes and shapes. On the other hand, the Fraunhofer approximation is applicable to large and/or opaque particles. This approach does not require optical properties for size distribution calculation, being for this reason, easier to use. However, it may lead to less accurate results, especially for small particles (below 50 microns in size) and/or for transparent particles [49].

The measurement time for LD depends on the sample size, particle size distribution, and dispersion accessory used. A monomodal material with a narrow particle size distribution can be adequately captured in few measurements, while a material with a broad particle size distribution, may require a longer measurement time, to ensure a proper representation of coarser particles [49,51].

In some cases, certain samples, such as pigments, can absorb light in the blue spectrum, causing a biased measurement during the blue light part of the measurement sequence. To mitigate this issue, the blue light measurement can be disabled, limiting the measurement to the main red laser, which is less prone to absorption by these pigments. Since red light has a longer wavelength (633 nm, e.g., from a Helium-Neon laser), it is less likely to be absorbed by most pigments, providing a more accurate particle size distribution measurement for such samples. However, this approach reduces the system sensitivity to smaller particles, as the blue light offers a better resolution for finer details in the particle size range. Therefore, disabling blue light (405 nm, e.g., from blue diode laser) may result in a trade-off between mitigating absorption effects and slightly reducing the precision when measuring smaller particles [49,51,52].

Another important parameter is the obscuration, which defines the conditions under which measurements can be initiated. Obscuration measures the percentage of emitted laser light that is lost due to scattering or absorption, providing an indication of sample concentration. Optimal obscuration settings depend on the sample and dispersion unit. As a rough guide, a range of 10–20 % is recommended for a wet dispersion unit [47,49].

Optimal conditions for the laser light diffraction measurements were optimized using FcCCD planning using JMP® 17.0 Software (Cary, USA). The statistical significance of the parameters was evaluated using Student's *t*-test (95 % confidence level, $\alpha = 0.05$) and ANOVA (p-value < 0.05). Based on the initial risk assessment analysis (Fig. 1 and Table 4), two CMVs were identified, namely stirrer speed and sonication power. Nine different method conditions were tested, for each one three replicates were conducted (Table 6 and Table 7).

Regarding the identified CMVs, stirrer speed is an important factor in laser light diffraction, as it directly affects particle size. Studies carried out by Kippax have demonstrated that stirrer speeds exceeding 2500 rpm ensure particle size stability, indicating that the entire sample is in suspension [47]. Below this speed, larger particles settle, while fine particles are disproportionately sampled. Additionally, the effect of sonication power has been investigated, revealing that excessive sonication can significantly reduce particle size, but as it may require a considerable time to reach stability, this aspect should be closely considered during method development [47,49].

All experiments were conducted in a randomized manner to minimize the influence of uncontrolled factors that could introduce bias to the response.

To assess the influence of each factor and their combinations on the responses, the polynomial coefficients were determined for the response (Fig. 2). A higher magnitude of the coefficient indicates a stronger main

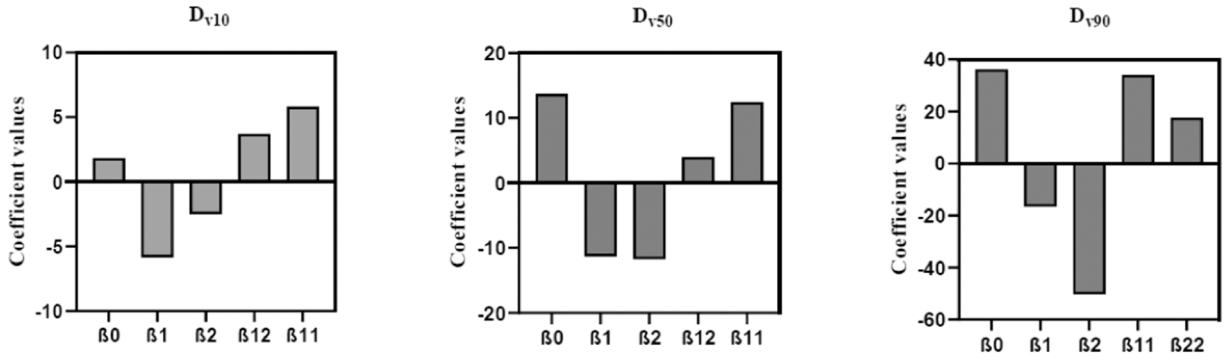

Fig. 2. Coefficient values of LD analysis extracted from mathematical models obtained from DoE. Results report to n=3. Key: LD: laser light diffraction; DoE: design of experiments.

effect on the system. Additionally, a positive coefficient means that there is an increase in the response, whilst a negative coefficient indicates that with an increase in this CMV, it will lead to a CAA decrease [53,54]. The integrated analysis of these responses resulted in distinct models. Fig. 3 displays the response surfaces and the corresponding contour plots for the D-values. A *t*-test analysis of coefficients indicates that, in the vast majority, parameters are highly significant, see Fig. S1. Moreover, Table S1 displays the coefficient terms estimated for the different responses, and respective statistical information.

The goal for all size distributions was to minimize the Dv_{10}, Dv_{50}, and Dv_{90} responses, indicating that the best value is the smallest possible, towards a stable and consistent measurement.

Accordingly, the increase in the CMV, sonication power and stirrer speed have a negative impact in all CAAs, having resulted in a reduction of particle size, as indicated by β_1 and β_2 coefficients. In what concerns the interaction term, the combination between CMV tends to revert the effect, leading to an increase in the particle size, although according to the respective magnitude (Dv_{10} and Dv_{50}), the result is not remarkably changed.

Aiming at selecting the more favorable settings for particle size determination, the desirability (*D*) function, described as the weighted geometric mean for several responses or value between 0 and 1 per response, was used. A non-zero value of *D* indicates that all responses are within the desirable range, while a value close to 1 indicates an optimal combination of the considered criteria (Fig. S1). A desirability value of 1 suggests that the response values are close to the target ones [54]. Additionally, Fig. 4 and Table S2 presents the interaction plots and the evaluation of ANOVA performed to assess model fitness respectively. Following the DoE experiments and data analysis, the desirability profiler suggests performing the LD analysis at 63 % for sonication power, and 3000 rpm of stirrer speed.

3.2.2. *Automated microscopy analysis*

When aiming to determine the "real particle size distribution", manual or automated microscopy analysis is the most accurate method, as it allows for the inspection of thousands of particles, ensuring confidence not only in the mean but also in the tails of the distribution [55]. By doing so, this method provides an enhanced statistical confidence in particle size determination, when compared to manual microscopy. Due to its accuracy, resolution, and high-throughput screening characteristics, this method is becoming increasingly recurrent in laboratories.

During the initial risk assessment and method development, various factors were investigated. These included sample preparation, smear, thickness, sample quantity, inclusion of the coverslip, illumination, and lens. In what concerns overall method development, there were several parameters which were tested, namely: (i) sample preparation, where the smear glass method was found to produce a thinner sample layer, compared to a tip spreading method; (ii) the use of coverslips, which did not significantly affect the analysis of sample; (iii) the choice of illumination strategy, which was dependent upon the sample characteristics. In order to improve particle visibility, episcopic (top light) should be employed for opaque substrates, whilst diascopic (bottom light) is preferable for transparent particles.

For particles below 3 μm, imaging was conducted using a 50x magnification objective lens, and a Sharp Edge segmentation was employed. Morphological filtering techniques, such as convexity and solidity filters, were applied to remove partially imaged or overlapping particles from the raw image data [41]. Touching particles and incomplete images were filtered out using specific area (pixels < 1000 and > 7000), circularity (< 0.8), and elongation (> 0.2).

Three distinct representative regions (Fig. 5) were carefully selected to maximize the number of imaged particles and ensure that the predefined criterion of 5000 counted particles per area was met according with ISO 13322–1 2014. This setting provided an adequate description of the sample distribution [20,41].

The Morphologi software provides several particle size parameters as volume or number distributions, including aspect ratio, and circle equivalent (CE) diameter. Additionally, particle shape parameters such as convexity, elongation, high sensitivity (HS) circularity, and solidity can be used to characterize particle shape.

By analyzing multiple zones on the glass slide, any potential variation or heterogeneity in the particle size distribution within the sample could be identified. This analysis helps to ensure that the measurements are representative of the overall sample, and that are not influenced by confined variations.

A case study is presented below where three glass slides were prepared from the same batch and three areas of each glass slide were analyzed as shown in Fig. 5. The variability was evaluated based on both size and shape parameters. CE diameter (by volume distribution) was considered for discussion and further outcomes of automated microscopy analysis included dendrogram and trend plot tools. This approach aimed to assess the precision of particle size analysis across different areas of the same sample.

A summary of the three glass slides (namely, glass slide 1 records R1, R2 and R3; glass slide 2 records R4, R5 and R6; and glass slide 3 records, R7, R8 and R9) in terms of the mean value of the size and shape parameters measured (by volume) is shown in Table 8. Fig. 6A depicts the parameter variability, where the length of the bars is proportional to the variability between the glass slides for the selected parameter, in this case CE diameter. It is possible to verify that there is low variability and, therefore, high similarity, across these glass slides, which is in agreement with the results shown in Table 8 and with the CE distribution profiles (Fig. 6B).

The CE diameter is calculated by converting the pixels of the two-dimensional image into a circle with an equivalent pixel area, thereby determining the CE diameter [56]. The parameter HS circularity quantifies how close a particle shape resembles a perfect circle, where a value of 1.0 indicates a perfect circle, and values closer to 0 correspond to

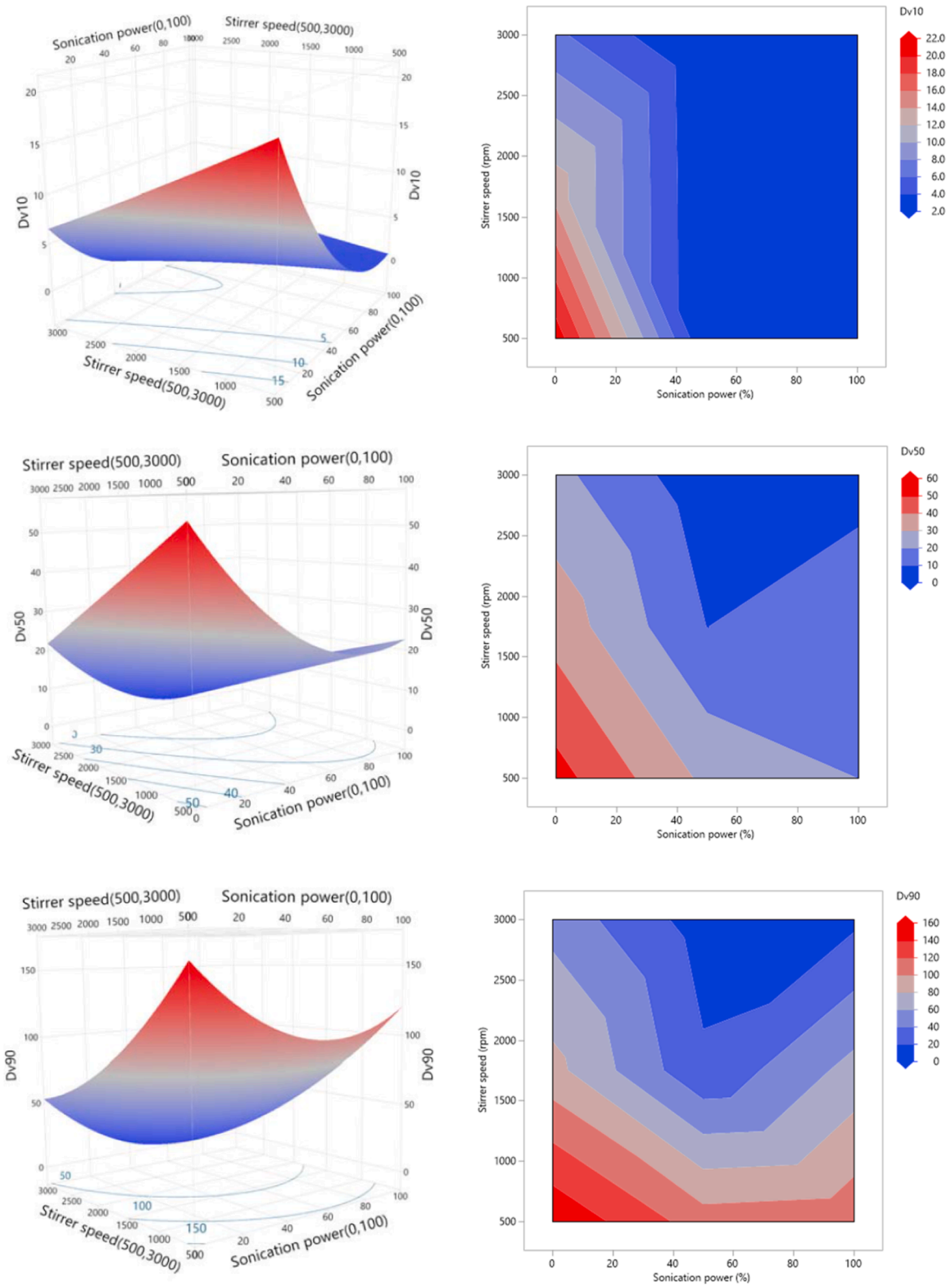

Fig. 3. Three-dimensional response surface and respective contour plots reflecting the effect of stirrer speed and sonication power on D values. Key: stirrer speed (rpm) (-1) 500, (0) 1750, and (1) 3000; sonication power (%) (-1) 0, (0) 50, and (1) 100.

elongated shapes [57]. As shown in Table 8, HS circularity values range from 0.72 for Dv_{10} to 0.93 for Dv_{90}, with higher values indicating more spherical particles at large sizes. The variability (RSD) decreases as the particle size increases, suggesting that smaller particles exhibit more shape variation, while larger particles are more uniformly spherical.

The aspect ratio provides the width-to-length ratio of a particle, describing its elongation. Values range from 0 to 1, with a rod-like particle having a value closer to 0 and circular particle approaching 1. In contrast, circularity measures the ratio of the circumference of a circle (with the same projected area as the object) to the object perimeter, where a value of 1 indicates a perfect circle [56]. According to the results, the aspect ratio remains consistent across all particle sizes, with

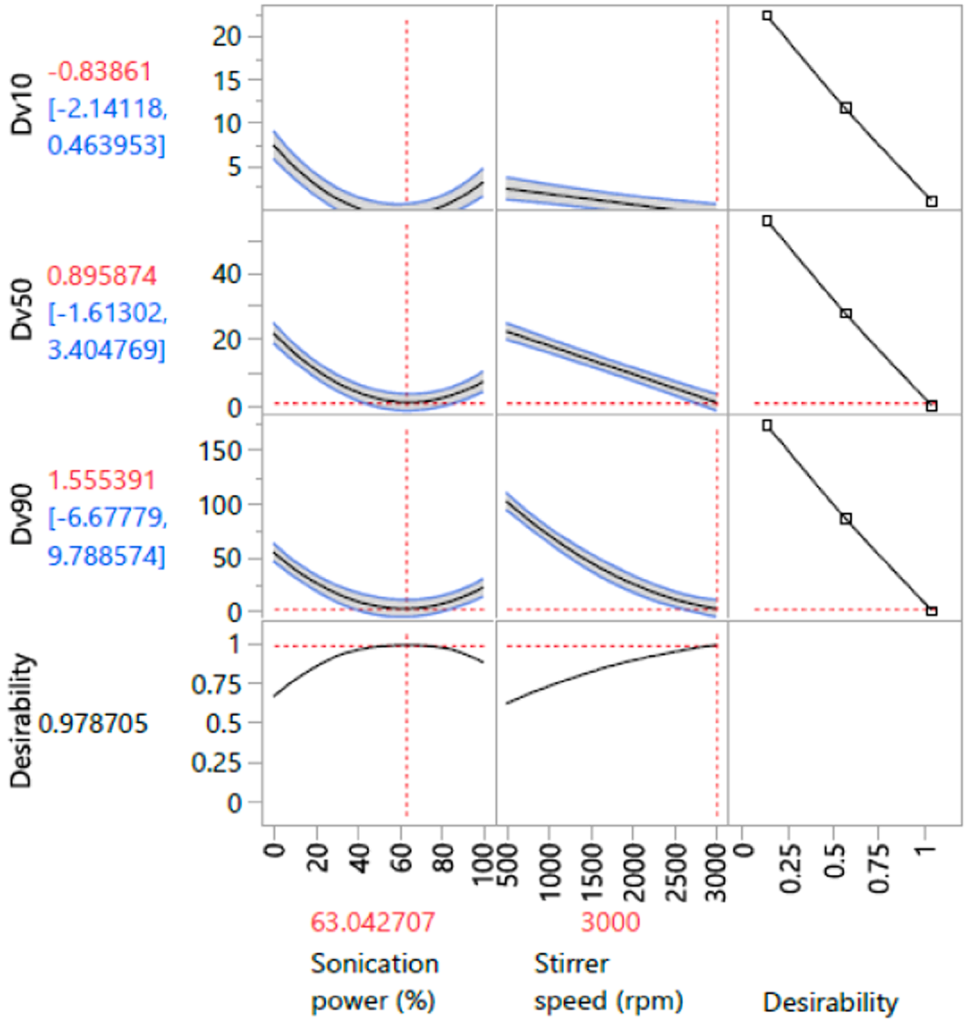

Fig. 4. Overall desirability for LD analysis optimization, according to the target imposed per CMV from DoE. Results report to n=3. Key: LD: laser light diffraction; CMV: critical method variable; DoE: design of experiments.

Fig. 5. Glass slide with clobetasol propionate 0.5 mg/g cream exhibiting three distinct representative regions for the automated counting of particles.

minimal variability, indicating a uniform width-to-length ratio and consistent particle shape. The circularity results suggest that smaller particles exhibit more shape variation, while larger particles are more uniformly spherical. Elongation results show greater variability for smaller particles, but this stabilizes at large sizes (Dv_{90}), indicating that larger particles tend to be less elongated.

The parameter solidity measures the density of a particle, with a value of 1 indicating a solid, smooth object, and values less than 1 indicating irregular boundaries or interval voids. Convexity reflects the surface roughness of a particle, where a value of 1 represents a smooth surface, and lower values indicate more irregular or "spiky" surfaces [56]. Both solidity and convexity show extremely low variability, particularly for large particles. The RSD values are negligible for these parameters, further emphasizing the uniformity of the particle shapes across different zones on the slides.

Table 8
Summary of mean measurements of size and shape parameters by volume distribution of three glass slides. Results report to a n = 3 ± SD.

Parameter variability	D_{10} Mean ± SD	RSD (%)	D_{50} Mean ± SD	RSD (%)	D_{90} Mean ± SD	RSD (%)
CE diameter (µm)	1.51 ± 0.06	3.68	2.17 ± 0.11	4.85	2.96 ± 0.09	3.14
HS circularity	0.72 ± 0.05	6.34	0.86 ± 0.03	3.80	0.93 ± 0.01	1.29
Aspect ratio	0.81 ± 0.00	0.36	0.87 ± 0.01	0.95	0.95 ± 0.00	0.43
Elongation	0.04 ± 0.00	9.67	0.13 ±0.01	6.60	0.18 ± 0.00	1.60
Solidity	0.91 ± 0.02	2.27	0.97 ± 0.01	0.00	0.99 ± 0.00	0.00
Convexity	0.94 ± 0.01	1.13	0.98 ± 0.00	0.42	0.99 ± 0.00	0.08

Key: HS: High sensitivity circularity. SD: standard deviation.

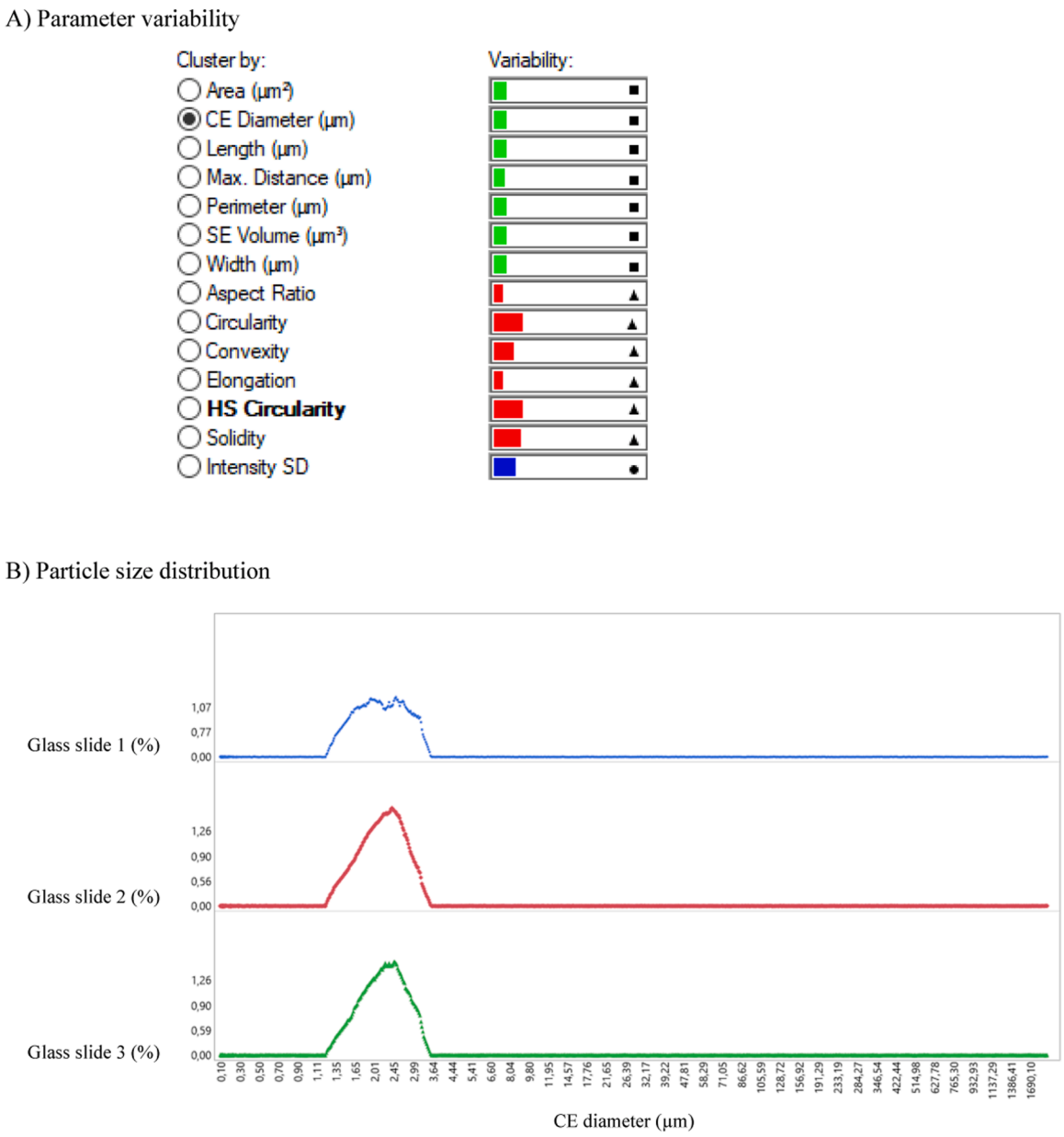

Fig. 6. Mean particle size (Circle Equivalent diameter by volume) of three glass slides. A) Parameter variability analysis, which depicts the variability of fourteen parameters. Accordingly, green bars represent size parameters (■), red bars represent shape parameters (▲), and blue bars related to light transmission (●). B) CE equivalent diameter distribution shown in Table 8. These demonstrate a high similarity in the CE diameter of the particles size.

The Dv_{10} values show some variation across the three slides, with glass slide 2 having slightly higher values compared to glass slides 1 and 3. On the other hand, Dv_{50} and Dv_{90} indicate that particle size distribution is consistent across the three glass slides, with low variability and high reproducibility. This is crucial for ensuring reliable and predictable performance of the semisolid formulations.

Morphological results for the same samples were also compared in terms of their shape distributions using the hierarchical cluster analysis (HCA) presented in Fig. 7. The links on the HCA represent the clustering of the most similar records together. As the HCA progresses, these clusters are further grouped into larger clusters. The x-axis of the HCA indicates the variation between glass slides, while the y-axis represents the Euclidean distances between clusters. A value close to 1 signifies a more divergent range of morphological values, indicating that the distribution curves of the groups do not overlap significantly. On the other hand, a value closer to 0 suggests a higher convergence, indicating that the groups have similar morphological characteristics. In this case, the distinct clustering patterns reveal that certain glass slides have similar particle size distributions. Clusters formed with distances around 0.05–0.1 are relatively homogeneous, while distance values around 0.1–0.2 suggest moderate differences. Slides in this range are somewhat similar but exhibit some variations. These differences could stem from sample preparation, measurement conditions, or inherent variability in the samples.

The trend plot (Fig. 8) displays the mean values selected from the CE diameter obtained for each glass slide. This plot can be used to assess statistical process control information. It provides an overview of how the selected parameter varies across different glass slides, allowing for the identification of trends and potential variation in the data. The plot shows four distinct trends, each represented by different colors (green, blue, and brown/light blue lines).

The CE diameters vary, in general, from 2.13 µm (R6) to 2.33 µm (R1). Particles represented by the blue lines show more variability in sizes, whereas those represented by light blue and brown lines show

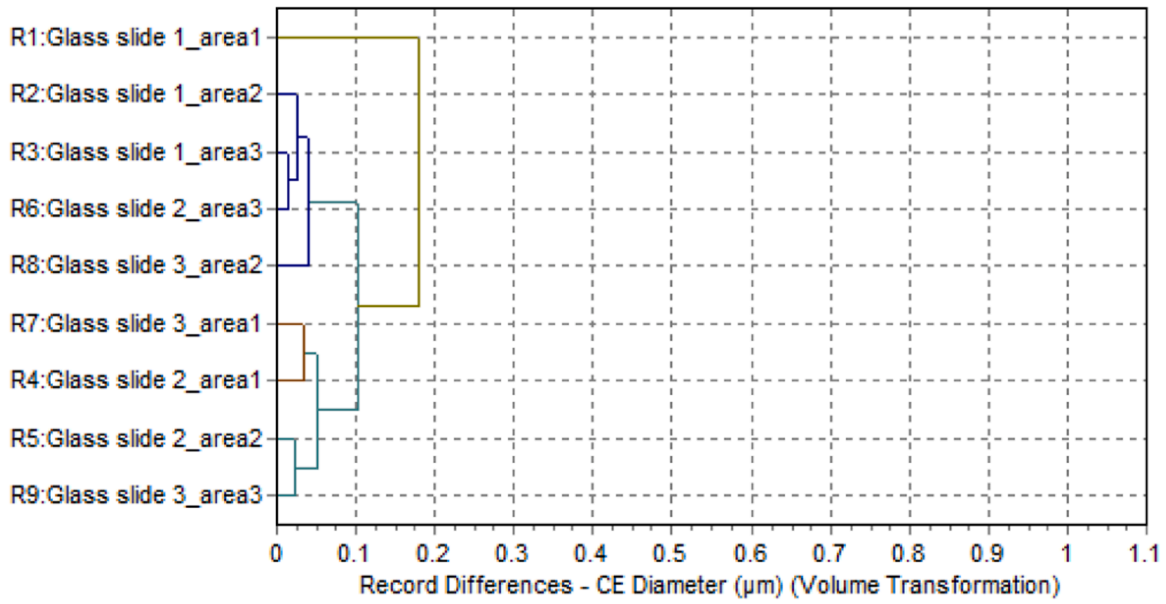

Fig. 7. Hierarchical cluster analysis (HCA), using the Euclidean distance, for the three glass slides taking into consideration the Circle Equivalent (CE) diameter.

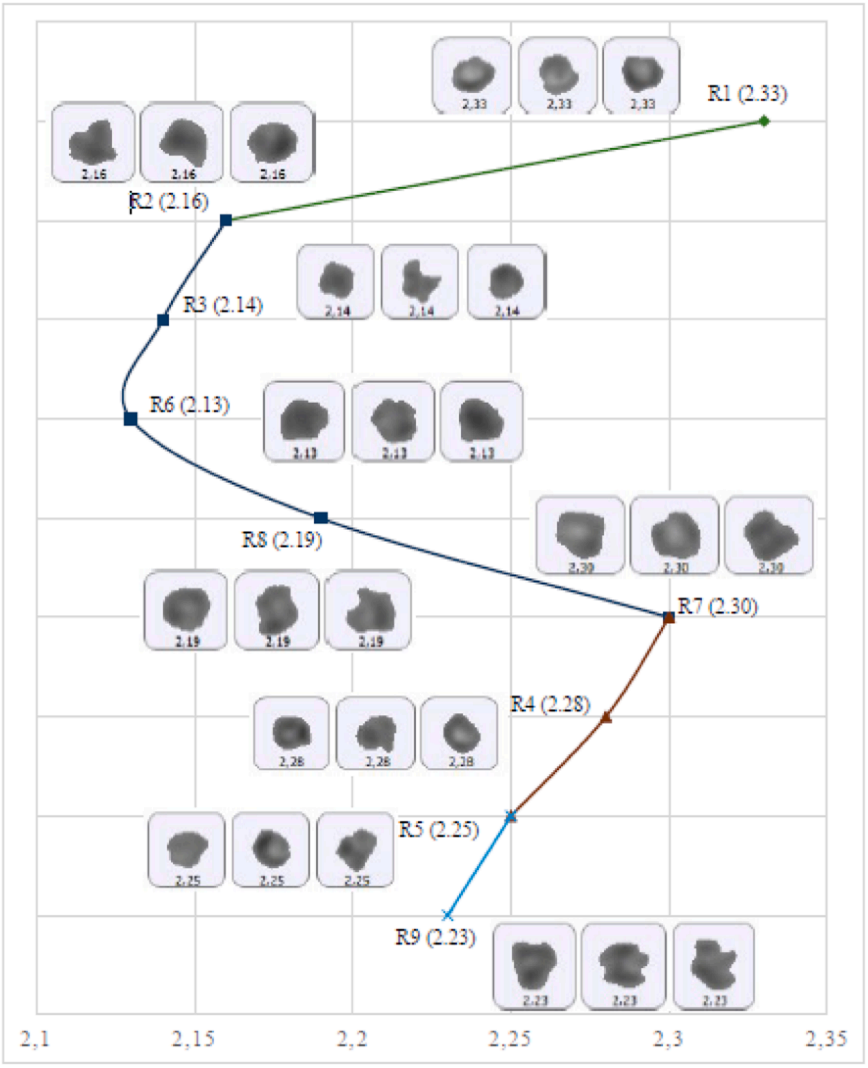

Fig. 8. Trend plot showing Circle Equivalent diameter between the samples, along with example particle images (µm). Green line: record 1, blue line: records 2, 3, 6 and 8, brown line: records 4 and 7, and light blue: records 5 and 9.

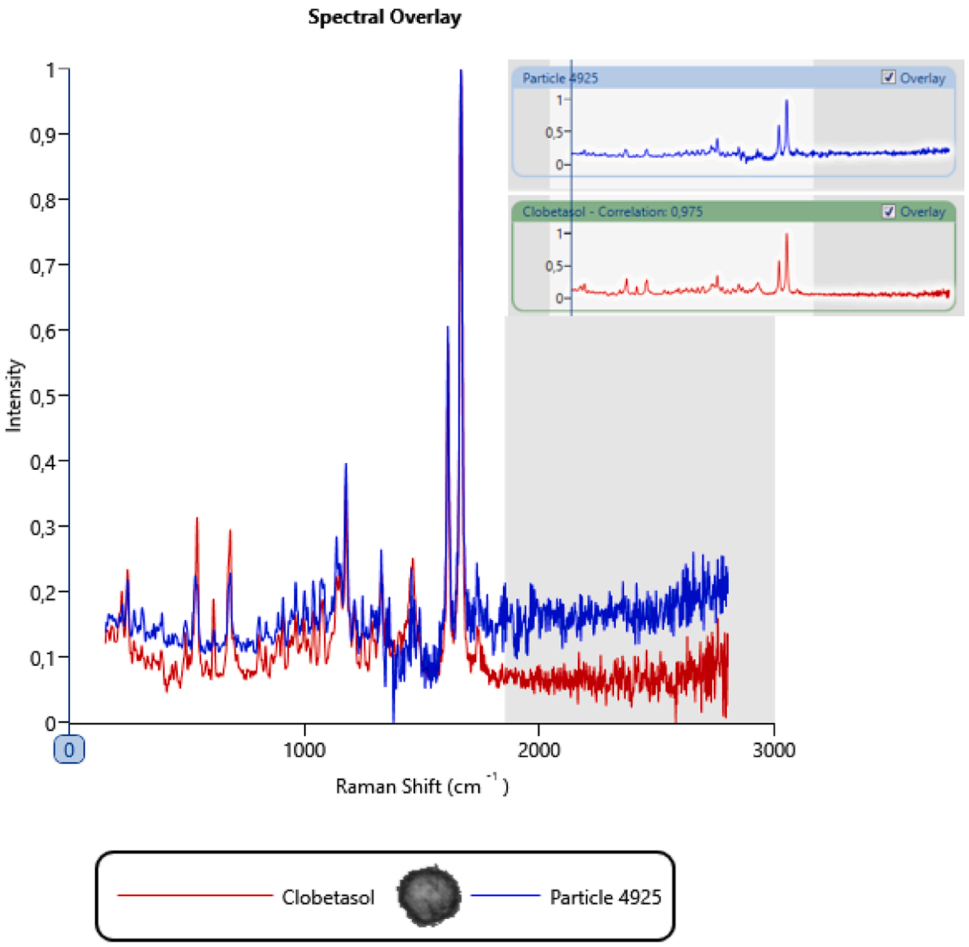

Fig. 9. Raman spectra collected of clobetasol spectral library and clobetasol cream product.

more consistency within their groups. R1 (green line) stands out as the largest diameter, indicating a potential need for further investigation into its measurement or sample preparation.

Overall, this study confirms the robustness and reproducibility of the particle size and morphology measurements across different zones of the glass slides. The combination of size and shape parameter analysis, hierarchical clustering, and trend analysis provides a comprehensive understanding of the sample heterogeneity. This is crucial for ensuring the reliable performance of semisolid formulations in pharmaceutical applications, where the consistency in particle size distribution directly affects the product stability, efficacy, and quality.

3.2.2.1. Chemical identification. Based on the previous knowledge obtained from AM, Raman spectroscopy was employed for chemical identification of particles. In this study 74 particles with CE diameters larger than 1 μm were specially targeted for analysis. Raman spectra were collected using a Raman laser operating at 30 % power, with an acquisition time of 7 seconds.

To identify the chemical components, i.e., API (Clobetasol propionate, CP) and excipients (chlorocresol, glyceryl stearate, cetostearyl alcohol, citric acid, sodium citrate, propylene glycol, and beeswax), a reference library with Raman spectra was created. Each formulation particle (droplet or solid particle) measured by Raman spectroscopy was compared to the reference spectra in the library, aiming to inspect an in-depth analysis of the distribution of the API in the formulation.

Fig. 9 shows the overlapped spectra, where a correlation value is displayed for each selected spectrum compared to reference spectra. The correlation values range from 0 to 1, where 1 represents a perfect correlation and 0 represents no correlation. In the case of the spiked sample, aiming at obtaining a formulation containing drug particles in suspension, a correlation value of 0.975 was obtained, indicating a high degree of similarity between the measured spectrum and the respective reference for CP. In contrast, the lower intensity or even absence of peaks corresponding to the API in the CP formulation indicates that the drug is mostly dissolved in the lipid droplets of the formulation, as displayed in the heatmap in Fig. 10. Each heatmap bar visualizes the chemical similarity between the particles in formulation A and spiked formulation B. In general, the heatmaps show varying levels of similarity for each component. The green areas emphasize where the particles in both formulations are highly similar, while the white areas show lower similarity. Some components, like CP and chlorocresol, show significant differences between formulations A and B (indicating more white areas), while others, like beeswax and cetostearyl alcohol, have almost complete similarity (indicating by large green areas).

All the outcomes before mentioned point out to the use of AM as a valuable technique for several purposes: i) identifying significant morphological parameters that distinguish between “good” and “bad” batches; ii) ensuring the consistency of product manufacturing process across all morphological parameters; and iii) identifying the most discriminatory morphological parameters for a specific product.

4. Method validation

In the AQbD approach, the final step is to ensure that the necessary control is in place and that the method is validated. Regulatory guidance documents such as USP <429>, EP chapter (2.9.31.) ISO 13320:2020 and ISO 13322–1:2014 provide general guidance as to the precision (repeatability and intermediate precision) that particle sizing methods

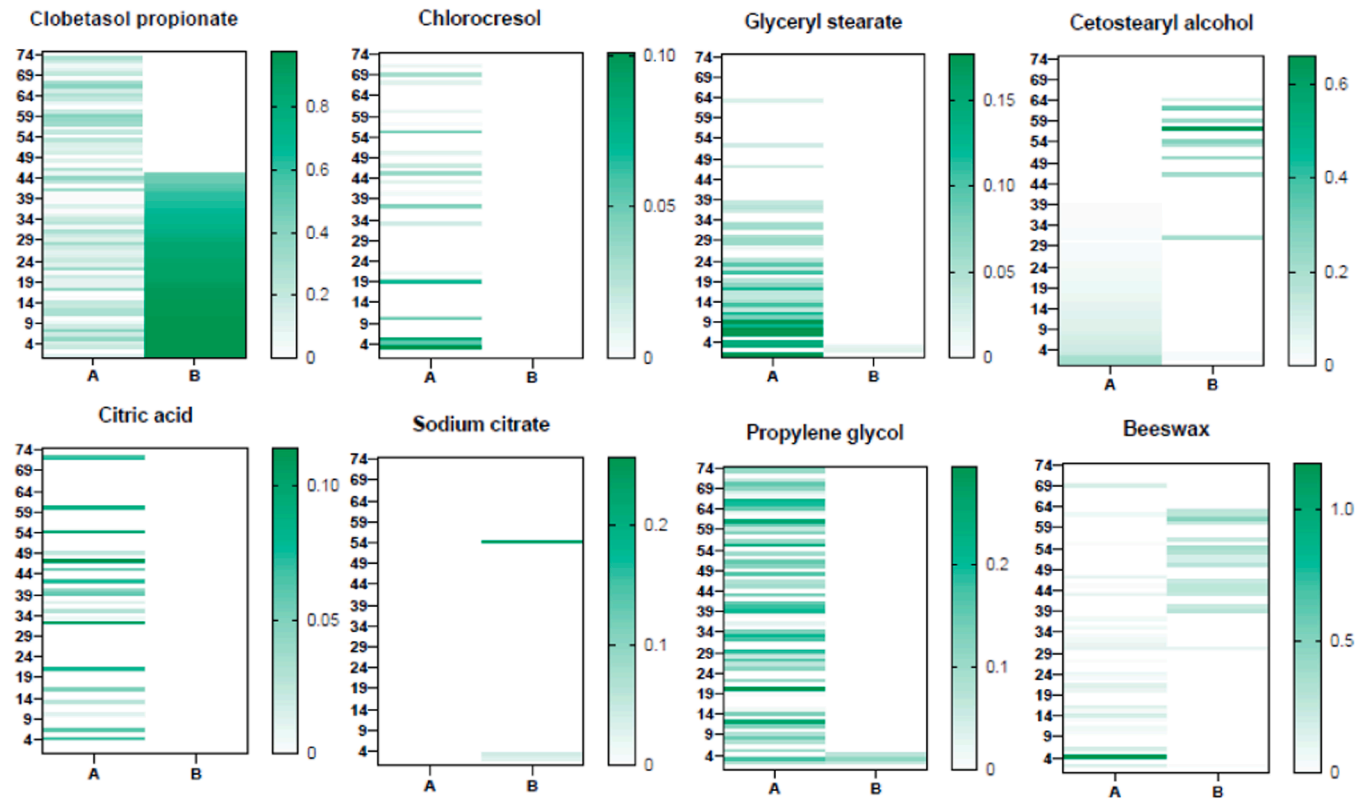

Fig. 10. Heatmap representing the correlation in terms of the chemical similarity of the particles measured and the respective components (API and excipients) of the formulation: A) formulation and B) spiked formulation. Green indicates high similarity and white low similarity. The YY-axis reflect the number of particles analyzed for chemical identification (74 particles identified per formulation).

Table 9
Repeatability and intermediate precision results of CE diameter (μm) from LD analysis. Results report to n=6.

CAAs	Repeatability		Intermediate precision	
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
Dv_{10}	1.85 ± 0.02	1.28	1.83 ± 0.04	2.12
Dv_{50}	6.05 ± 0.33	5.52	6.26 ± 0.36	5.74
Dv_{90}	13.80 ± 0.36	4.15	13.73 ± 0.48	3.48

Key: CE: circle equivalence; LD: laser light diffraction; CAAs: Critical analytical attributes.

Table 10
Repeatability and intermediate precision results of CE diameter (μm) from AM analysis. Results report to n=6.

CAAs	Repeatability		Intermediate precision	
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
Dv_{10}	1.51 ± 0.12	8.24	1.49 ± 0.10	6.39
Dv_{50}	2.04 ± 0.08	3.72	2.02 ± 0.10	5.00
Dv_{90}	2.91 ± 0.07	2.40	2.88 ± 0.11	3.97

Key: CE: circle equivalence; AM; automated microscopy; CAAs: Critical analytical attribute.

should deliver. However, these specifications are relatively broad and may not always address specific requirements of the product/process. It is crucial to ensure that the precision of the particle sizing method matches the requirements of the ATP for the specific product. The ATP defines the desirable performance characteristics of the analytical method, taking into consideration the intended use, product specifications, and quality requirements.

The repeatability and intermediate precision on both laser light

Table 11
Particle size distribution of CE diameter (μm) from laser light diffraction measurements. Results report to an n=3.

	D values	Mean ± SD	RSD (%)	Acceptance criteria (RSD%) *
Batch 1	Dv_{10}	2.19 ± 0.02	1.00	≤ 15
	Dv_{50}	6.64 ± 0.33	4.90	≤ 10
	Dv_{90}	15.86 ± 0.71	4.49	≤ 15
Batch 2	Dv_{10}	2.13 ± 0.02	0.85	≤ 15
	Dv_{50}	5.82 ± 0.09	1.55	≤ 10
	Dv_{90}	17.73 ± 0.28	1.59	≤ 15
Batch 3	Dv_{10}	2.37 ± 0.02	1.02	≤ 15
	Dv_{50}	6.92 ± 0.11	1.59	≤ 10
	Dv_{90}	18.83 ± 1.22	6.47	≤ 15

Key: *USP chapter <429>, below 10 μm, these values must be doubled.

Table 12
Particle size distribution of CE diameter (μm) from automated microscopy measurements. Results report to an n=3.

	D values	Mean ± SD	RSD (%)	Acceptance criteria (RSD%)*
Batch 1	Dv_{10}	1.45 ± 0.17	11.45	≤ 15
	Dv_{50}	2.19 ± 0.19	7.32	≤ 10
	Dv_{90}	3.08 ± 0.10	3.39	≤ 15
Batch 2	Dv_{10}	1.56 ± 0.22	13.82	≤ 15
	Dv_{50}	2.23 ± 0.25	11.40	≤ 10
	Dv_{90}	2.96 ± 0.34	11.65	≤ 15
Batch 3	Dv_{10}	1.65 ± 0.18	10.73	≤ 15
	Dv_{50}	2.41 ± 0.19	7.69	≤ 10
	Dv_{90}	3.15 ± 0.08	2.55	≤ 15

Key: *USP chapter <429>, below 10 μm, these values must be doubled.

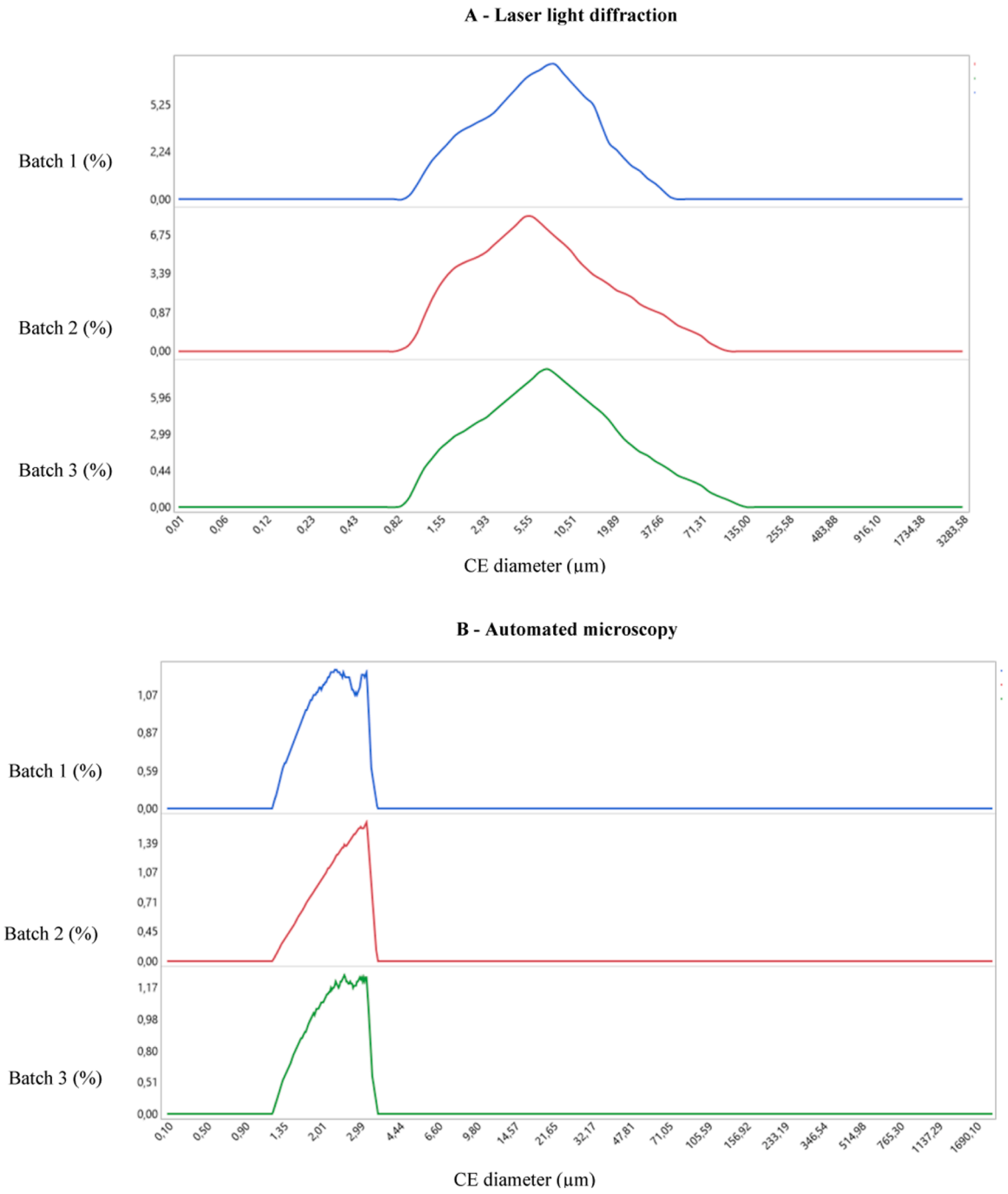

Fig. 11. Comparison of particle size distribution profiles of the batches 1, 2 and 3 obtained from LD (A) and AM (B) measurements.

diffraction and automated microscopy analyses were evaluated using six replicates with three measurements each. The intermediate precision study involved another analyst and was conducted on a different day within the same laboratory. The results of the particle size analysis using laser light diffraction and automated microscope analysis are presented in Table 9 and Table 10, respectively and the corresponding particle size distributions are displayed in Fig S2. The reported results are expressed in CE diameter by volume distribution for AM and volume density for LD, both providing information on the volumetric size distribution of the particles.

The good precision demonstrated by both the LD and AM methods, with RSD values below 10 %, confirms the reliability of these techniques for particle sizing in this context. Thus, the method validation process has ensured that both the LD and AM methods are suitable for delivering precise and consistent particle size measurements that meet the requirements outlined in the ATP. Also, these data reinforce the need of using both techniques to infer on an accurate particle size analysis, whenever possible.

5. Looking ahead

Particle size plays a critical role in the pharmaceutical industry and needs to be carefully considered in various aspects. In product development, particle size directly impacts the absorption rate and bioavailability of the API. It is crucial to control the particle size to ensure optimal absorption and therapeutic efficacy [58].

Additionally, particle size also affects the flow behaviour of pharmaceutical products, which has implications for production, storage,

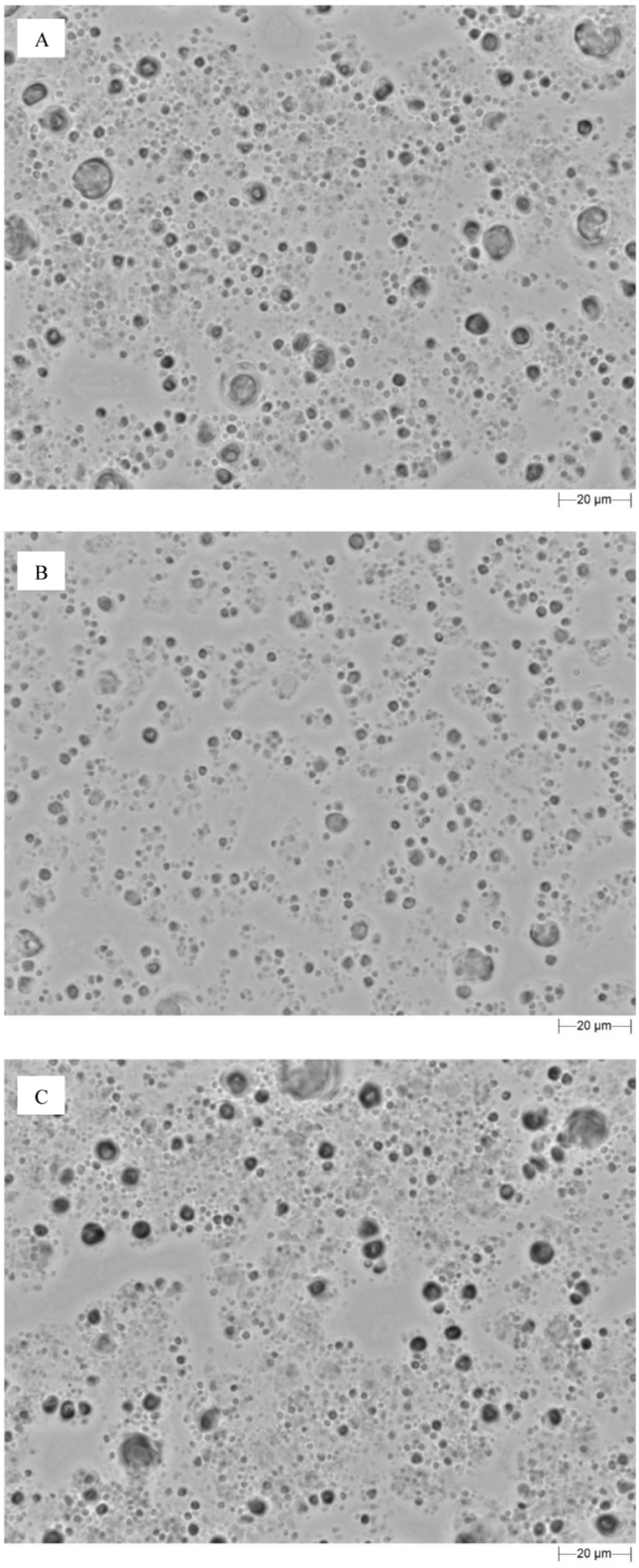

Fig. 12. Automated microscopy analysis. A) batch 1. B) batch 2. C) batch 3.

transport, and packaging properties of formulations. Controlling the particle size is essential to prevent manufacturing issues and ensure compliance with quality control standards [59].

In this study, three commercial semisolid batches of CP cream formulations were analyzed using the validated techniques described. The particle size distribution results are presented in Table 11 and Table 12.

Regarding laser light diffraction analysis, the Fig. 11 shows that the particle size distributions of the three commercial batches appear to be similar. On the other hand, micrographs obtained from automated microscopy analysis (Fig. 12) for the same batches display different appearances, although the particle size results were very similar. The discrepancy in the images could possibly be attributed to variations in the glass slide preparation. Note, however, that all the reported results are in conformity with the acceptance criteria and indicate that both methods could be considered suitable for application in manufactured product or quality control analysis. Such trend is consistent with the perspective shared by Grubbs and co-workers, who reinforce the need to use more than one method to investigate the particle size and shape analysis of distinct samples. Their recommendation underscores the need for consistency in the chosen method for characterization, as variations in data can occur regardless of the selected technique [60].

In another study performed by Prestes *et al.* emulsified systems were analyzed in order to understand the influence of the internal phase droplets (concentration, size and morphology) characteristics on the systems physicochemical attributes. The authors were able to conclude that these properties can be altered not only by the formulation components but also by the analytical methodologies employed. Microscopic analysis was performed manually by transferring the formulations onto a glass slide and covered them with a coverslip. For LD, wet sample dispersion was used, with the samples dispersed in purified water and homogenized in an ultrasound bath prior to analysis, maintaining an obscuration level of up to 8 %. The authors concluded that both methodologies could not be compared, as they yielded statistically different results for the same sample [61]. This conclusion is consistent with other literature findings, which also reported that microscopic analysis is not directly comparable to the LD technique. Accordingly, the techniques showed general agreement; however, both the area and volume size distributions determined by the optical microscopy were greater than the volume-weighted distribution determined by LD. It is believed that these differences can be attributed to the effect of the third dimension on the measurement of particle size [62].

These studies consistently demonstrate that particle size analysis often produces differing results depending on the technique used. This aligns with the findings of the present research, where discrepancies between AM and LD were also observed. Despite these differences, both techniques are valuable to study particle characteristics such as size, shape and chemical composition. Their complementary nature enables a more comprehensive assessment of particle size in semisolid dosage forms. Ultimately, this emphasizes the need for a tailored approach to particle size analysis, especially in complex formulations where multiple variables come into play.

6. Conclusions

Setting specifications based on product understanding is a crucial aspect of AQbD approach, being an essential step to assure an effective quality control within the pharmaceutical industry. Particle size is often identified as a critical quality attribute of the product, as it directly impacts the performance of product. Therefore, in the pharmaceutical industry, a tight control of particle size is deemed necessary, alongside the development of suitable specifications together with methods capable of providing a reliable assessment of this attribute.

In the present work, development and validation studies concerning laser light diffraction and static image methods were carried out in accordance with the ICH Q2 (R2) guideline. Through risk assessment and factorial design tools, in the laser light diffraction methodology, the impact of sonication and ultrasound, on the method responses, was evaluated to determine the optimal setting for particle size assessment. For automated microscopy, only a risk assessment was deemed necessary to provide a comprehensive application of the technique and meet the ATP requirements. Both approaches enabled a precise particle size measurement. Also, it is highlighted the need of a trade-off between the

techniques and their complementarity from a particle size by design perspective: while LD offer higher throughput and more precise values at the cost of peak resolution and broadening, AM has an intrinsic more variability, but a more reliable information in what concerns the size-shape analysis.

The validated methods successfully demonstrated the implementation of an AQbD methodology in defining particle size methods. This approach ensures that the particle size distribution of materials is well-controlled, improving production workflows and resulting in a higher quality end product with desired bioavailability, and validated effectiveness required for drug product manufacturing.

In conclusion, an effective control of the particle size distribution contributes to the reproducible production of high-quality pharmaceutical products, that meet the required specifications for optimal performance and therapeutic efficacy.

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CRediT authorship contribution statement

Lucas Chiarentin: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Carla Vitorino:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Margarida Miranda:** Writing – review & editing. **Fabio Major:** Software, Resources, Methodology. **José Catita:** Writing – review & editing, Resources. **Vera Moura:** Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.colsurfa.2024.135679.

Data availability

Data will be made available on request.

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