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Description and evaluation of a semi-automatic system for particle size analysis by microscopy

Edward E. Sims and Roger Withington *

Roche Products Ltd., P.O. Box 8, Welwyn Garden City (U.K.)

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Summary

A projection microscope, microprocessor-controlled digitizing platen and a desk-top computer have been combined to provide a system for rapid, objective particle size analysis by microscopy. Computer software has been written to enable the particle size distribution of acicular and non-acicular particles to be determined according to the protocol described in British Standard 3406, Part 4. The function of the system has been tested by determining the particle size distribution of samples of Sephadex G25 (Fine), spray-dried lactose and an acicular drug substance. Results of replicate determinations by several operators demonstrate that the method is rapid, reproducible and free from the operator bias associated with subjective assessment of particle size.

Introduction

The microscope has been used for particle size analysis for a number of years, and is referred to (Allen, 1981) as an 'absolute method' because individual particles are observed and measured. All methods of particle size measurement have a number of advantages and disadvantages in their use, which may frequently restrict their application. Microscopy is no exception, and British Standard 3406, Part 4 (1963), presents a standard test method in an attempt to reduce inaccuracies and variability.

Some disadvantages of particle size analysis by microscopy may be associated with subjective assessment of projected area diameter and operator tedium and fatigue due to observation of large numbers of particle images. Several methods have

* Present address: Merrell Pharmaceuticals Ltd., Rusham Park, Whitehall Lane, Egham, Surrey, U.K.
Correspondence: E.E. Sims, Wyeth Laboratories, Huntercombe Lane South, Taplow, Maidenhead, Berkshire SL6 0PH, U.K.

been developed to increase accuracy and reduce operator error, but may either remain tedious (e.g. image splitting techniques) or involve the purchase of high cost equipment. A relatively inexpensive system for a semi-automatic method of particle size analysis by microscopy was demonstrated (Withington and Peters, 1980) which combined the essentials of the British Standard method with an image analysis system. This paper describes the equipment and computer software and presents the results obtained for some size analyses of typical materials.

Materials and Methods

Equipment

The equipment consists of a microscope fitted with a projection facility, a digitizing platen, a microprocessor measuring unit and a desk-top computer. The microscope (Carl Zeiss, Oberkochen) is illuminated by a 250 W light source, and is fitted with an Abbé condenser, objectives of magnification ×6.3, ×10 and ×40, a beam splitting prism, a projection/extension tube, eyepiece lens (×12.5) and a beam rotation mirror. The optical system allows projection of a suitable image of the sample to the digitizing platen situated adjacent to the microscope. The magnifications available at the platen for the ×6.3, ×10 and ×40 objectives are ×200, ×340 and ×1280, respectively.

The platen consists of a glass plate under which is a square mesh of fine, magnetized wires connected to a MOP-1 microprocessor unit (Kontron). Electrical impulses are sent along the wires starting alternately from each of two adjacent sides of the grid (the X and Y axes). Each electrical pulse causes a slight physical displacement of the wires (magnetostriction) which moves along the wires at a constant speed of 5 $m \cdot s^{-1}$. Because the wires are magnetized there is a corresponding movement of their magnetic field which induces a current in the receiving coil of the stylus, which is also connected to the microprocessing unit. The time elapsed between the generation and interception of each pulse is measured by the microprocessor control unit and is used to calculate the distance of the stylus from the X or Y axis. Time and distance measurements on successive pulses allow the X, Y co-ordinates of the stylus position to be determined. The area enclosed by an outline traced on the digitizing platen is calculated from the continuously evaluated X, Y co-ordinates of the stylus position. The microprocessor converts co-ordinate data to area (mm^2) enclosed by the traced outline. For acicular materials, size distributions are determined by particulate length and the microprocessor is programmed to interpret the co-ordinate data by determining the distance (mm) between two discrete points obtained when the extremities of a projected particle image are touched with the stylus.

The Hewlett Packard 9815A desk-top computer is interfaced with the MOP-1 microprocessor enabling automatic sizing, counting, and classification of particles together with a degree of control over the size analysis procedure through feedback, via a printer, to the operator. The HP 9815A is programmed to output number and/or weight distributions, but because weight distributions are generally of

greater importance than number distributions for pharmaceutical powders, data is collected in a way that takes into account the greater influence of large particles on a weight distribution. Particles are sized and classified by area into size classes corresponding to the series recommended by the British Standards Institution. (B.S. 3406, Part 4). This series expresses particle size as the diameter of the circle with the same projected area as the particle, based on a $\sqrt{2}$ progression above and below 53 μm. The method for the size analysis of acicular particles is based on length measurements. The same size classes as the projected areas are used to describe the size distributions. Some further features of B.S.3406, Part 4, were used in the design of the semi-automatic method. A minimum requirement for the counting of 100 'large' particles was incorporated to ensure that the conditions governing the determination of weight size distributions are met with respect to the minimum number of particles required for the control size class. Other features of the British Standard method incorporated into the HP 9815A software include corrections for numbers of fields and field areas observed at different magnifications, the calculation of accuracy factors during analysis, and the provision of a separate procedure for acicular particles which do not fall within the general scope of the method. Practical recommendations referring to sample preparation were also observed, and the size distribution calculations were based on the B.S. method. By using the combination of objectives the equipment may be reliably used for complete particle size analysis over the range of projected area diameters of 2.34–149.90 μm (corresponding to B.S. size classes 4–15). For the analysis of acicular materials, the smallest particle length that may be sized is approximately 2.4 μm, enabling a length size distribution with a lower limit of B.S. size class 4.

General method (Fig. 1)

Preliminary scan

The whole slide is scanned and the outline of what appear to be the largest particles are traced. The computer identifies the largest particle, determines the British Standard size class to which it belongs and instructs the operator to select the most appropriate objective for accurate sizing of the largest particles.

Sizing the largest particles

The largest particles are defined, for the purpose of analysis, as those belonging to the 3 largest size classes present in the sample. Using the objective indicated by the computer the operator traces the outline of obviously 'large' particles on the slide. The area of each particle is calculated and the size class to which it belongs is determined. Particles belonging to the 3 largest classes are counted into the appropriate class. Smaller particles are rejected by the computer (they will be sized and counted at a higher magnification). There is a special case for counting when only the $\times 40$ objective is used. Because no greater magnification is available particles are classified into all possible classes but the total number of particles in the largest 3 classes are monitored. When 100 'largest' particles have been classified counting continues at this magnification but only into the classes smaller than the largest 3

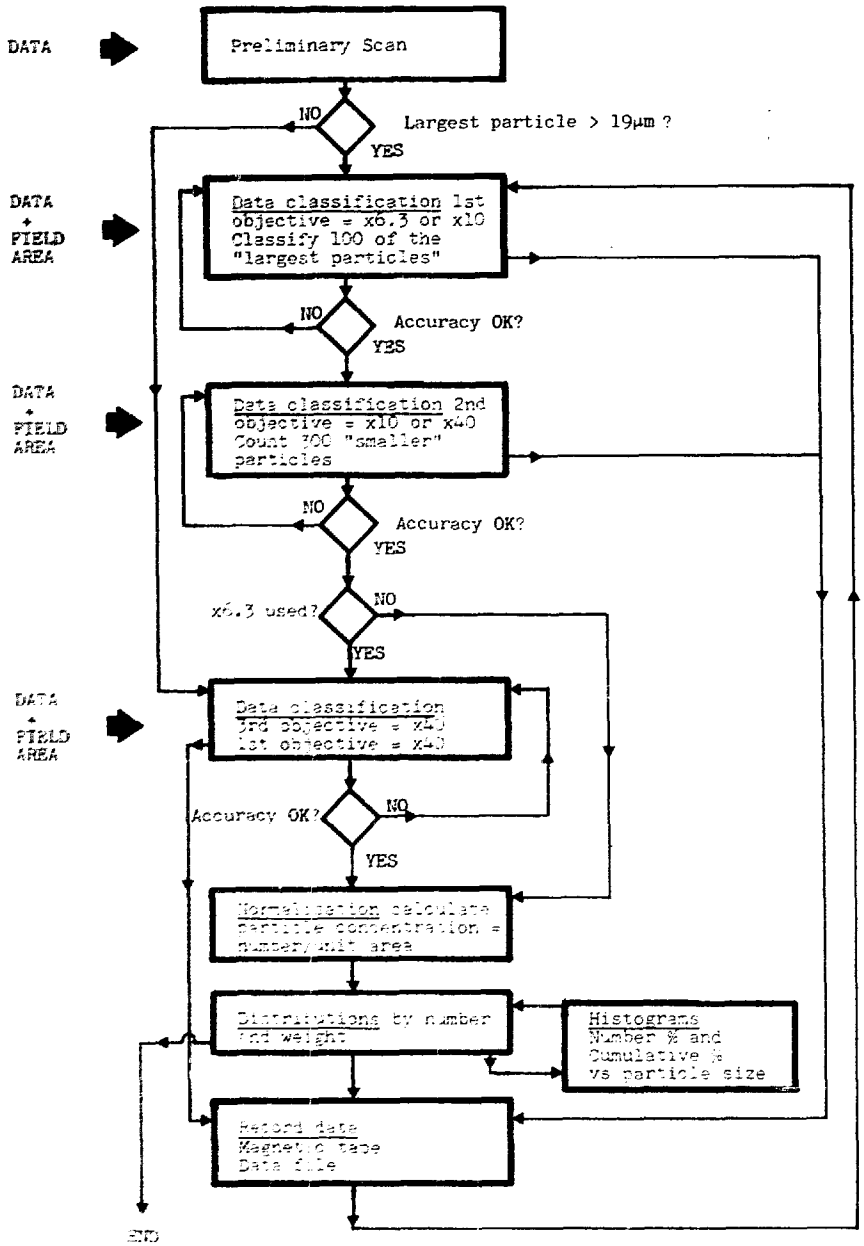


Fig. 1. Programme structure for particle size analysis by microscopy for classification by projected area diameter.

until a total of 400 particles have been classified. During counting the number of fields in which particles are sized are recorded by manual operation of an event counter connected through the MOP-1 microprocessor to the computer.

Sizing the smaller particles

When 100 large particles have been sized and classified the operator may select a programme routine which calculates accuracy factors for the particles counted

according to the British Standard method. If the accuracy factors are unsatisfactory counting must continue at the last magnification with periodic retesting of the accuracy factors. If the accuracy factors are satisfactory the computer prompts the operator to select the next highest magnification. Sizing and counting are continued as before. Particles large enough to have been counted at the previous magnification are rejected by the computer. Particles smaller than can be sized accurately at this magnification are counted as 'undersized' particles and, if necessary, will be sized and counted at the next magnification.

Sizing is continued until a total of 300 particles have been classified, or counted as undersize. The operator may then check the accuracy factors as before; if the factors are satisfactory the operator is informed of the total number of particles counted and the percentage of particles that are undersize (by number). If this second magnification is the ×10 objective the operator can either end the analysis or continue sizing at the next higher magnification. In the latter case sizing and counting are performed in essentially the same manner as before until a total of 300 particles have been classified at ×10 and ×40. Once again accuracy factors may be checked to ascertain that a sufficient number of particles have been classified.

Data reduction

When sizing is complete the operator selects a computer programme that converts the raw data into particle size distributions. The raw data consists of the number of particles counted into each size class at each magnification and the appropriate field counts. Because different numbers of fields will have been examined at each magnification the data is first 'normalized' to a common field number. The data can then be output as: (i) number distribution; (ii) % number distribution; (iii) cumulative % undersize by number; (iv) % weight distribution; and (v) cumulative % undersize by weight, as selected by the operator. In addition the computer calculates the particle sizes (diameter of the circle with the same projected area) below which there are 90%, 84%, 50%, 16% and 10% of particles in the sample. These percentiles may be used for rapid comparison of the median particle size and the coarse and fine tails of the distribution.

The general method is summarized in Fig. 1.

Method for acicular particles (Fig. 2)

For the purpose of this method of size analysis by microscopy, acicular particles are defined as those having a length : breadth ratio equal to or greater than 3.14 (π). The basis for this approach is that as a rectangular particle goes from square to acicular (i.e. increasing length : breadth ratio) the size class to which it is assigned by length moves from: (i) the same class as would be assigned if measured by area, to (ii) the next largest class when length : breadth ratio reaches $1/2\ \pi : 1$, to (iii) the next largest class again when length : breadth ratio reaches $\pi : 1$. This sequence is repeated for each $1/2\ \pi$ increment in length : breadth ratio.

For this method, it was considered that when a difference of two size classes occurs (i.e. for a length : breadth ratio of $> \pi$) between the two methods of

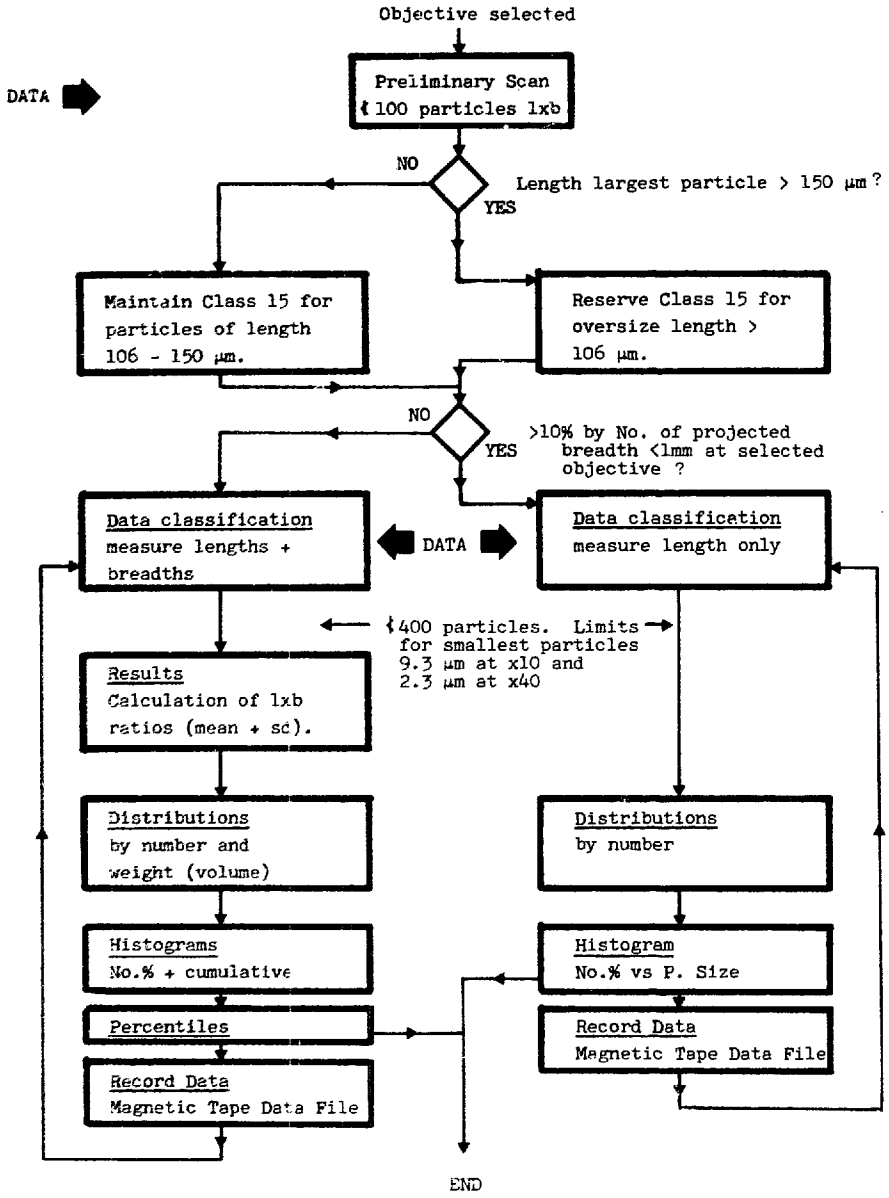
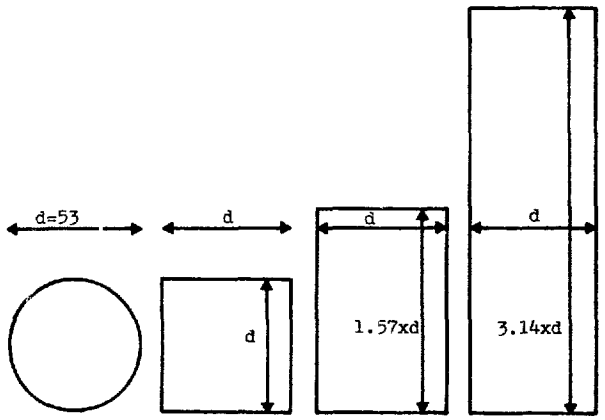


Fig. 2. Programme structure for particle size analysis of acicular materials by microscopy for classification by particle length.

measuring size then the particles should be recognized as acicular, and classified by length and not area. This is explained diagrammatically in Fig. 3. The general approach to particle size analysis of acicular particles is the same as for non-acicular particles. Particles are sized by measuring their length rather than their projected area. For straight-sided rectangular or cylindrical particle's length and breadth are easy to identify. Irregularly shaped particles must be considered to be enclosed by the smallest possible rectangle and the length and breadth of the rectangle measured, as shown in Fig. 4. Measurements are made by touching the points A, B, C and D



Size (area) = diameter of sphere with same projected area	53	59.8	75	106
BS size class	12	13	13	14
Size (length)	53	53	83	166
BS size class	12	12	14	16
Size (breadth) eg. sieving	53	53	53	53
BS size class	12	12	12	12

Fig. 3. Relationship between different size measurements (μm) for spherical and non-spherical particles.

with the measuring stylus. When there is any doubt about whether or not particles are acicular a computer programme is available to make a preliminary scan on length : breadth ratio. Measured particles are classified by their length into the British Standard series of size classes. Their breadth is used to calculate the length : breadth ratio. On the assumption that the particles have a square cross-section and are of equal density, breadth can also be used to calculate particle volume and relative weight. The breadth of the projected microscope image of some particles may be smaller than 1 mm. Such dimensions cannot be measured accurately with the measuring stylus so only the length of the particle is measured and the length : breadth ratio and weight distribution cannot therefore be calculated.

Preliminary check

A preliminary check may be required to determine the percentage of acicular

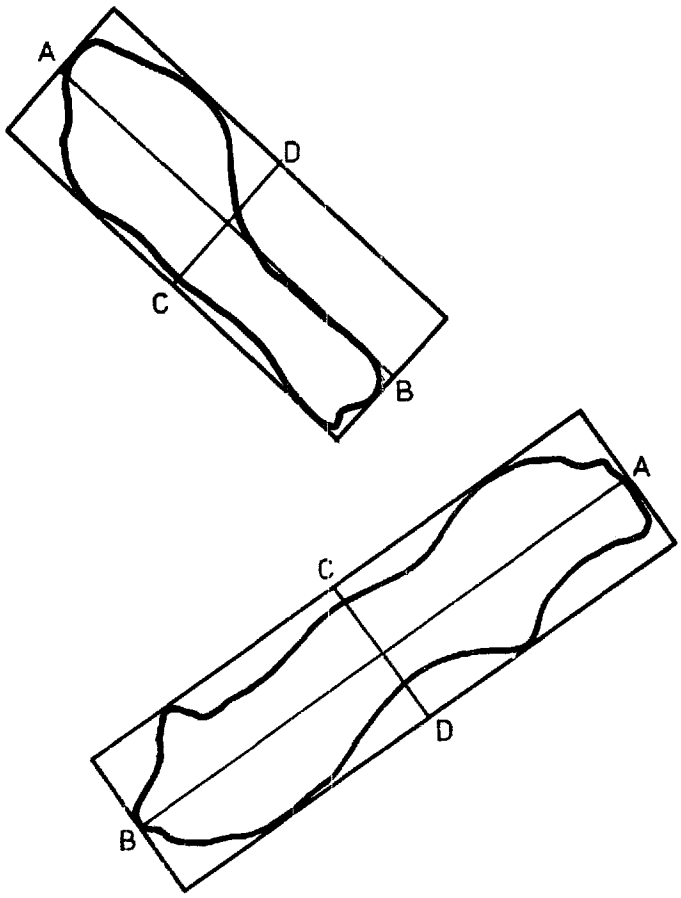


Fig. 4. Measurement of acicular particles.

particles, and therefore give an indication as to which size analysis procedure is applicable (standard or acicular).

Preliminary scan

The breadths of at least 100 particles taken at random from the whole slide are measured. If more than 10% of the particles have a breadth of less than 1 mm at the selected magnification, then size analysis will be done by length only. The lengths of the largest particles present are also measured. The computer determines whether or not the largest particles lie in a size class above B.S. size class 15. If they do it is necessary to use class 15 as an oversize class for all particles larger than class 14.

Particle size analysis

Size analysis is performed using either the $\times 10$ or $\times 40$ objective. The length and breadth, or length only (according to the preliminary scan result), of at least 400 particles are measured. Length and breadth may be measured in either order, by touching the ends of the relevant dimension with the stylus. The measured length

and breadth must be at right angles to each other. A summary of the method for acicular materials is shown in Fig. 2.

Data reduction

The type of size distributions that can be obtained are determined by whether or not particle breadth has been measured. When length and breadth measurements have been made the same types of distribution are calculated as for non-acicular particles. The calculation of weight distributions assumes that particles have a square cross-section and uses particle volume (length × breadth^2) as the weighting factor. In addition to particle size distribution the mean and standard deviation of all particle breadths and of length : breadth ratios are calculated. When only length has been measured, weight distribution and length : breadth ratios cannot be calculated.

Materials

The equipment and programme function were tested by performing particle size analyses on the following samples:

Sephadex G.25 (Fine) (Pharmacia)

Spray dried lactose, sieved, < 75 μm (DMV, Veghel, The Netherlands)

Spray dried lactose, sieved, < 106 μm (DMV, Veghel, The Netherlands)

Ro 31-1118/001, sieved, < 250 μm (a cardioselective β-blocker; Roche).

The samples were chosen for their range of particle sizes and particle shapes. Sephadex G.25 (Fine) consisted mainly of spherical particles of narrow size range. The spray-dried lactose samples presented wider ranges of particle size and shape. Particles of Ro 31-1118/001 were predominantly acicular.

Preparation of samples

The Sephadex G.25 (Fine) was used as received, and the other materials were sieved as indicated. Subdivision of samples was effected by chute splitters (Endecotts) down to 1 g. A rotating mini-riffler (Roche Engineering) was used to obtain samples of 100 mg.

Suspensions were prepared from the 100 mg samples and made up to approximately 5 ml using a small magnetic stirrer and low energy ultrasonics when necessary. A teat pipette was used to withdraw a quantity of this suspension and this was transferred to a second container where further gradual dilution was effected to produce a suspension of adequate concentration for particle size analysis. Periodic checking by microscopy was used to determine when the desired dilution had been achieved. In all cases, 1% sorbitan mono-oleate (Span 80, Honeywill-Atlas) in light liquid paraffin (Fisons Scientific) was used as the dispersion medium. In addition, *n*-hexane (Fisons Scientific) was used to aid dispersion of Ro 31-1118/001.

Suspensions were sampled by pipette and transferred to a counting chamber (modified Fuchs-Rosenthal ruling, ADH) and allowed to settle for at least 30 min before analysis. A counting chamber was necessary for all determinations; the grid rulings were used to define the boundaries of individual sizing fields and enabled reference to the total area examined at each magnification via an event counter

connected to the microprocessor. Analyses were performed on separate slides of the prepared suspension of appropriate dilution.

Results and Discussion

Particle size distributions obtained by individual operators for each material were presented in both tabular form as relative frequency distributions (B.S. size class vs percentage in class by number and weight) and graphical form (cumulative weight percentage undersize vs particle size upper limit of B.S. size class). These methods of presentation allowed direct comparison of results, in order to identify potential sources of error due to operator variation, sample preparation or sampling. Statistical methods of testing particle size distribution data have been reviewed by Herdan (1960), including the *t*-test, the chi-squared test and more complex analysis of variance. In order to assess the magnitude of the variability between operators for Sephadex G.25 (Fine) and spray-dried lactose (< 106 μm), the *t*-test was used to compare that means of percentile data obtained by each operator on replicate samples of the materials.

Sephadex G.25 (Fine)

Six slides of separate suspension sample of Sephadex G.25 (Fine) were analyzed by one operator to assess the reproducibility of the equipment and the method of sample preparation. There was good agreement between the results for the 6 slides over 98% of the weight distribution as shown by the mean values for the size distributions with their corresponding standard deviations in Table 1. Parity be-

TABLE 1

SIZE DISTRIBUTIONS BY WEIGHT OF 6 SAMPLES OF SEPHADEX G25 (FINE) DETERMINED BY ONE OPERATOR TO DEMONSTRATE REPRODUCIBILITY OF ANALYSIS

B.S. size class	Size class upper limit (μm)	Mean weight percent in size class	Standard deviation
15	149.9	0	0
14	106.0	26.6	2.5
13	75.0	56.1	3.9
12	53.0	15.0	2.2
11	37.5	1.6	0.7
10	26.5	0.1	0.1
9	18.7	0	0

Percentiles	90	84	50	16	10
Mean size (μm) (n = 6)	93.9	86.6	65.8	52.5	46.6
Standard deviation (μm) (n = 6)	1.3	2.1	0.7	1.4	1.4
Coefficient of variation (n = 6) (%)	1.4	2.4	1.1	2.7	3.0

tween samples is also reflected by the low values for the standard deviations in the percentiles, which are derived from the cumulative weight percentage size distribution curves. Additional slides of separate riffled samples were analyzed by 3 inexperienced operators, and the results were grouped according to operator, and tested statistically against the results for the size distributions calculated from Table 1. The *t*-test was used to systematically compare the mean 90, 50 and 10 percentiles to assess differences between operators. All results subjected to this statistical treatment gave *t* values of less than 0.8 for all percentiles, which is not significant. A summary of the size distribution data is shown as a cumulative weight size distribution curve in Fig. 5.

Spray-dried lactose (< 75 μm)

The spray-dried lactose was sieved through a 75 μm sieve to obtain a finely sized material for evaluation. Four samples were analyzed by the same operator to test the reproducibility of the sampling technique and sample preparation (Table 2) and duplicate analyses were performed on two of the samples to check operator variability. The duplicate results were compared with the original analyses and showed little or no difference, and hence it may be reasonable to assume that differences between the size distributions in Table 2 to be primarily due to sampling. However, the cumulative size distribution curves by weight were in close agreement,

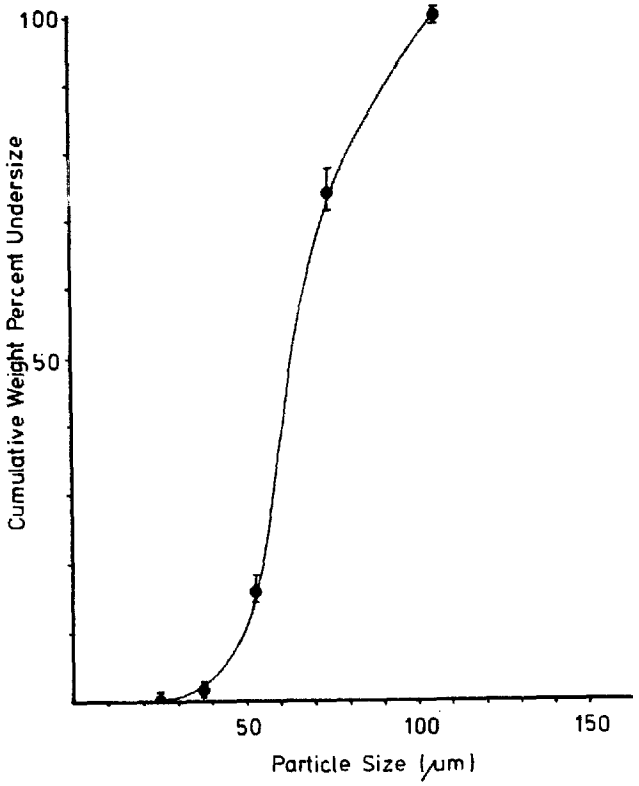


Fig. 5. Cumulative weight size distribution of Sephadex G25 (Fine) by projected area diameter. ⧫, mean and standard deviation for 6 slides taken from the same suspension sample, analyzed by a single operator.

TABLE 2

WEIGHT SIZE DISTRIBUTIONS OF 4 SAMPLES OF SPRAY-DRIED LACTOSE (SIEVED, < 75 μm) BY MICROSCOPY (1 OPERATOR, 4 SAMPLES)

B.S. size class	Sample no.				Mean (n = 4)	Standard deviation (n = 4)
	1	2	3	4		
15	0	0	0	0	0	0
14	17.5	28.0	27.5	30.2	25.8	5.7
13	41.5	42.1	34.5	30.4	37.1	5.7
12	17.8	13.9	24.2	24.1	20.0	5.0
11	15.8	9.3	7.2	5.7	9.5	4.4
10	3.7	3.8	3.8	4.9	4.0	0.6
9	2.1	1.8	1.4	2.3	1.9	0.4
8	0.9	0.6	0.9	1.3	0.9	0.3
7	0.4	0.4	0.3	0.5	0.4	0.1
6	0.1	0.1	0.1	0.4	0.2	0.1
< 6	0	0	0	0	0	0

Percentiles	90	84	50	16	10
Mean size (μm) (n = 4)	93.4	85.9	60.7	36.7	29.4
Standard deviation (μm) (n = 4)	3.4	5.5	2.3	2.9	2.0
Coefficient of variation (n = 4) (%)	3.6	6.4	3.8	7.9	6.8

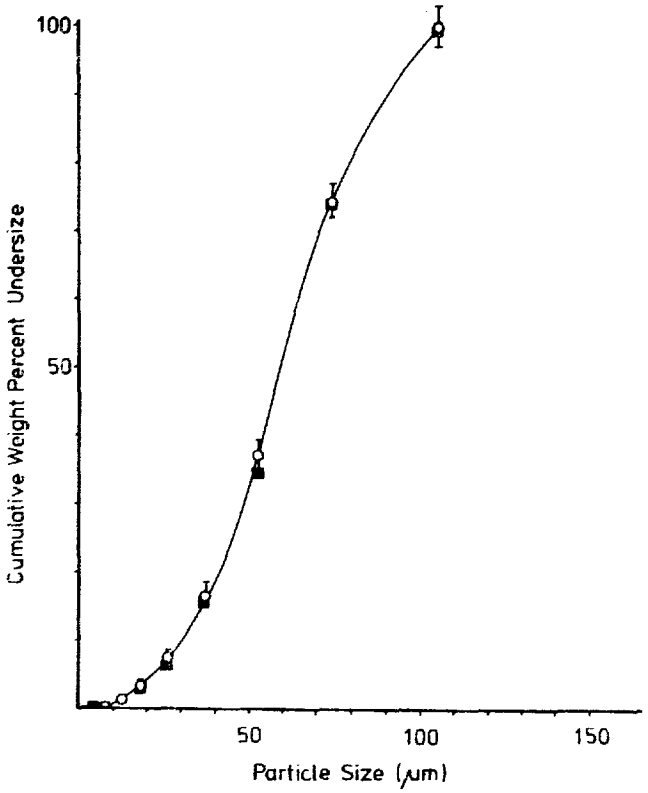


Fig. 6. Cumulative weight size distribution of spray-dried lactose (sieved, < 75 μm) by projected area diameter. $\bar{O}$, mean and standard deviation for 4 suspension samples analyzed by one operator. ■, size distribution of an additional suspension sample analyzed by a second operator.

as shown by the values for the mean and standard deviations for the percentiles of the 4 samples. The mean curve is shown in Fig. 6, and the result for an analysis of an additional sample by a second operator is included to support the conclusion that the method is relatively free from operator bias.

Spray-dried lactose (< 106 μm)

This material consisted of particles of greater range of sizes than the Sephadex G.25 (Fine) and 75 μm sieved spray-dried lactose. Four samples were analyzed by one operator and the percentage means and standard deviations for each size class were calculated for the weight distributions (Table 3). Comparison of the standard deviation with the mean value for each size class demonstrated less variation than that observed with the spray-dried lactose (< 75 μm) samples. The same operator was used for both materials and the major source of variation is attributed to sampling. Reproducibility was acceptable between samples analyzed by the same operator when individual results were compared. The effect of operator on reproducibility was investigated by comparison of the 90, 50 and 10 percentiles for the weight size distributions from Table 3 with the corresponding percentiles from single analyses of two separate suspension samples by a second operator. The percentiles were evaluated by a *t*-test; the values obtained for *t* were 1.80, 0.45 and 1.28, respectively. The table of *t* distribution showed *t* to be significant at $0.5 > P > 0.1$ for the 'tails' of the size distribution curves. Insignificant values for *t* were obtained for the 84 and 16 percentiles, showing insignificant difference between the size distributions obtained by the two operators over at least 68% of the size range. The cumulative size distribution curve for the spray-dried lactose (< 106 μm) by weight is shown in Fig. 7.

TABLE 3

PARTICLE SIZE DISTRIBUTIONS BY NUMBER AND BY WEIGHT FOR 4 SAMPLES OF SPRAY-DRIED LACTOSE (SIEVED, < 106 μm) BY 1 OPERATOR

B.S. size class	Size class upper limits (μm)	Mean percentage by weight in each size class (n = 4)	Standard deviation (n = 4)	Mean percentage by number in each size class (n = 4)
15	149.9	19.6	1.7	0.5
14	106.0	26.4	3.9	2.1
13	75.0	27.4	3.0	5.6
12	53.0	14.1	3.5	8.0
11	37.5	6.3	1.0	10.1
10	26.5	2.2	0.2	10.1
9	18.7	0.8	0.1	10.9
8	13.2	0.3	0.0	11.0
7	9.4	0.3	0.0	23.9
6	6.6	0.0	0.0	12.0
5	4.7	–	–	3.1
4	4.3	–	–	0.6
< 4	3.3	–	–	1.7

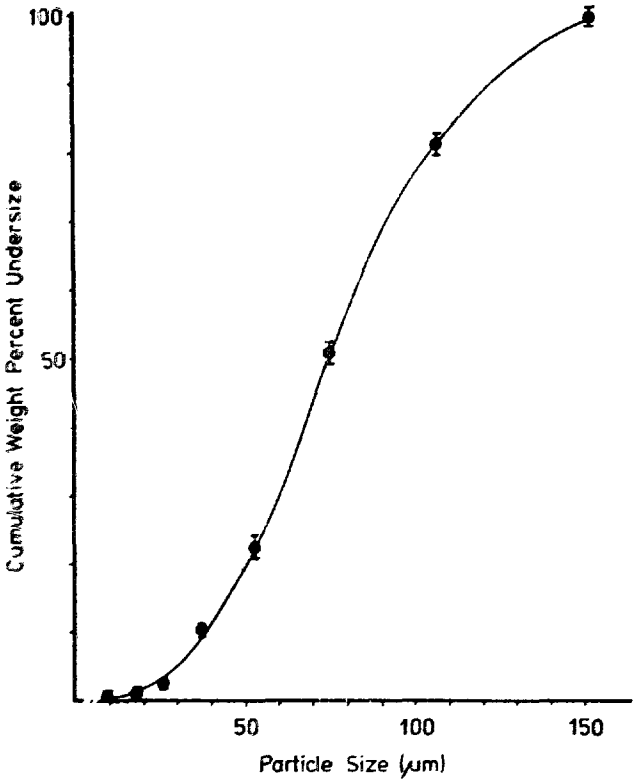


Fig. 7. Cumulative weight size distribution of spray-dried lactose (sieved $< 106\ \mu m$) by projected area diameter. ⧳, mean and standard deviation for 4 suspension samples analyzed by one operator.

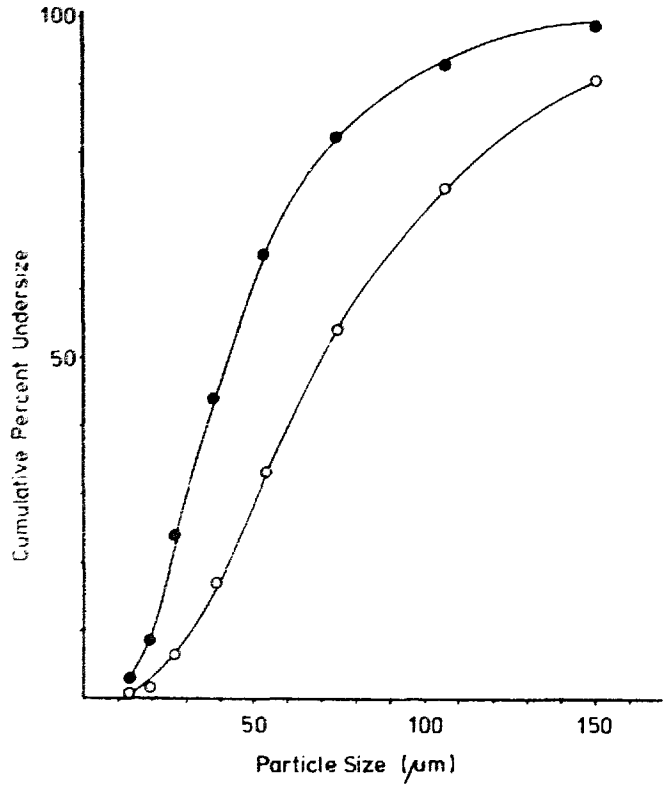


Fig. 8. Cumulative size distributions of an acicular material (Ro 31-1118/001) by particle length. ●, cumulative number size distribution; ○, cumulative weight (volume) size distribution.

Ro 31-1118/001

Initial microscopical examination of this material revealed the majority of the individual particles to be acicular. The particle size analysis programme for acicular materials was selected for length analysis and length and breadth measurements of individual particles were taken using the ×40 objective. Separate suspension samples were analyzed by two operators; duplicate determinations were performed on each sample. Variation between operators was minimal and the pooled results are presented in Fig. 8 as cumulative size distributions by number and by weight (volume) where particle size (length) is represented by the upper limits (μm) of the corresponding British Standard size classes.

Conclusions

A major disadvantage of microscopy for particle size measurement is operator variability, and the importance of this factor has been reported by Nathan et al. (1972). However, it may be concluded that the semi-automatic method is capable of producing reproducible results independent of operator, and the equipment as described offers improvement in speed of analysis and time involved in data reduction over manual methods.

The results show that the equipment may be reliably used for a variety of materials and the software written to ensure concordance with British Standard 3406, Part 4, maintains a standard procedure for the method of analysis. In most cases, number and weight distributions may be obtained to allow critical examination of the results so that spurious conclusions should not be drawn from a single odd result. It is well known that a major source of error or variation in any microscopical method of particle size analysis is in the sample preparation, and ensuring that a representative sample is obtained. This remains a disadvantage but its effect can be minimized by performing replicate analyses on carefully prepared samples. In this regard the semi-automatic system described here enables the particle size distribution of a number of replicates to be determined rapidly and provides flexibility in the method of data presentation for assessment.

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