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Estimation of the minimum uncertainty of DNA concentration in a genetically modified maize sample candidate certified reference material

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Abstract Homogeneity testing and the determination of minimum sample mass are an important part of the certification of reference materials. The smallest theoretically achievable uncertainty of certified concentration values is limited by the concentration distribution of analyte in the different particle size fractions of powdered biological samples. This might be of special importance if the reference material is prepared by dry mixing, a dilution technique which is used for the production of the new and third generation of genetically modified (GMO) plant certified reference materials. For the production of dry mixed PMON 810 maize reference material a computer program was developed to calculate the theoretically smallest uncertainty for a selected sample intake. This model was used to compare three differently milled maize samples, and the effect of dilution on the uncertainty of the DNA content of GMO maize was estimated as well. In the case of a 50-mg sample mass the lowest achievable standard deviation was 2% for the sample containing 0.1% GMO and the minimum deviation was less than 0.5% for the sample containing 5% GMO.

Introduction

Homogeneity testing is one of the most important steps of certification and production of certified reference materials (CRMs) [1]. The homogeneity of samples is calculated on two levels: within-bottle and between-unit. In the case of powdered samples the within-bottle homogeneity is often limited by the inhomogeneous distribution of analyte among the solid particles.

There is a trend to decrease the sample mass in modern analytical and bioanalytical techniques (e.g., 50 mg for polymerase chain reaction analysis); therefore, for powdered biological and environmental reference materials the determination of the minimum sample mass to be used for an analysis has increasing importance. ISO Guide 30 [2] contains terms and definitions used in connection with reference materials but this parameter is not defined there. The certification report of CRMs generally describes the suggested sample volume or mass to perform a certain method. The minimum sample mass can be defined as the lowest sample mass where the certification is valid with a given uncertainty. Pauwels and Vandecasteele [3] published a method for the calculation of this parameter for different CRMs. In this statistical approach the given values are strongly dependent on the method that is used for the determination of the certified parameters. If the analyte concentration is not the same in every particle of a solid sample, a decrease in the sample mass increases the theoretically available smallest deviation of measurement.

In the case of biological and environmental materials, the concentration of analyte very often differs in the different particle size fractions of a given material. A good example is the DNA content of ground maize powder as different parts of the maize kernel, like embryo, endosperm and seed coat, have different hardness, structure and DNA content.

In the production of powdered reference materials the particle size distribution of the sample is a crucial question as was reported by Kramer and coworkers [4, 5]. If the particle size distribution has two peaks or the size of the particles is larger than 100–150 μm, the sample can be separated during transport. Smaller particles (below 5–10 μm) can be charged and the sampling from the bottle becomes more complicated; therefore, small particles should not exceed a certain ratio. The ideal particle size distribution has only one peak and shows a maximum between 10 and 100 μm.

The third generation of genetically modified (GMO) CRMs are prepared using a dry mixing technique [6] in which the necessary amount of the milled non-GMO and GMO maize kernels are mixed with each other.

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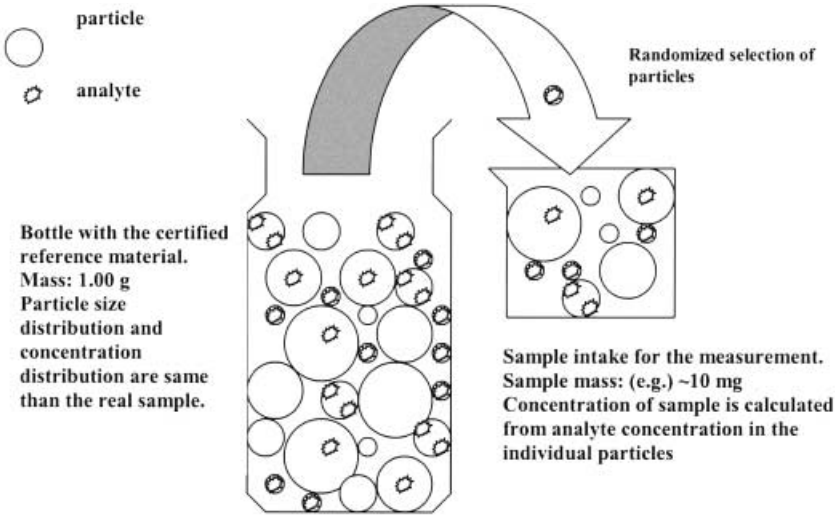

Fig. 1 Principle of model calculation. On the basis of the particle size distribution, the concentration distribution and the dilution ratio, 1.00 g reference material is simulated. Every individual sample has mass and concentration data, but the model does not consider the deviation within the particle size fraction. The sample amount required is constituted by random selection of individual particles

After measuring the particle size distribution and the concentration of analyte in the different particle size fractions of a candidate CRM, the relative standard deviation versus sample amount function can be calculated by the mathematical model developed.

Materials and methods

The mathematical model for the calculation of the sample amount – standard deviation function

By knowing the particle size distribution and the concentration of analyte in every particle size fraction a batch sample can be simulated with a mass equal to the one whole unit of GMO CRM (1.00 g). During the simulation of sampling, selection is done among the individual particles of the sample in a randomized way. The mass of the selected particles is summed, and the process is stopped when the mass of the sample is equal to or a little higher than the required sample intake (Fig. 1). In the case of PMON 810, the sample amount was varied between 0.05 and 50 mg. The concentration within such a sample can be calculated by dividing the summed amount of analyte in the individual particles by the total amount of sample. The standard deviation can be obtained by repeating this calculation.

A simple program was implemented in Borland Pascal with the Delphi 4.0 version. The calculated concentration data were imported to the Microsoft Excel program for the calculation of the relative standard deviation values and for the calculation of the corresponding function.

The program contains an additional unit which is able to take the real production process of the reference material into consideration, for example, the mixing of the GMO and non-GMO material at different ratios.

Data on accurate sample mass, the number of particles and the concentration of analyte in the individual sample are obtained as the result of a single run. Repeated running produces a standard deviation of each GMO DNA concentration. By repeating the calculation with different sample masses the standard deviation of the GMO DNA concentration related to the sample mass can be obtained.

Determination of particle size distribution

The particle size distribution was determined by a laser diffraction method with a personal-computer-controlled SympaTec Helos/KA instrument (Sympatec, Clausthal-Zellerfeld, Germany). The laser is a He/Ne laser, λ = 632.8 nm, beam diameter 20.2 mm and the beam extension is 16×. The diffraction was measured after suspending the sample in methanol. Thirty measurement points were obtained in the 0.5–800 nm range on a logarithmical scale.

Measurement of DNA content in the different particle size fractions

The milled maize kernel sample was separated into different particle size fractions by use of a sieve series. The DNA was extracted from each fraction using a NucleoSpin Plant genomic DNA isolation extraction kit (Macherey-Nagel, Düren, Germany). The DNA content of the extracted solutions was measured by spectrophotometry at 260 nm.

Application of the model

The application of the model is presented for three samples. The first, sample A, was prepared by grinding a maize kernel with a simple laboratory grinder (M20, IKA Labortechnik, Brussels). Sample B was prepared by grinding a maize kernel in a track mill.

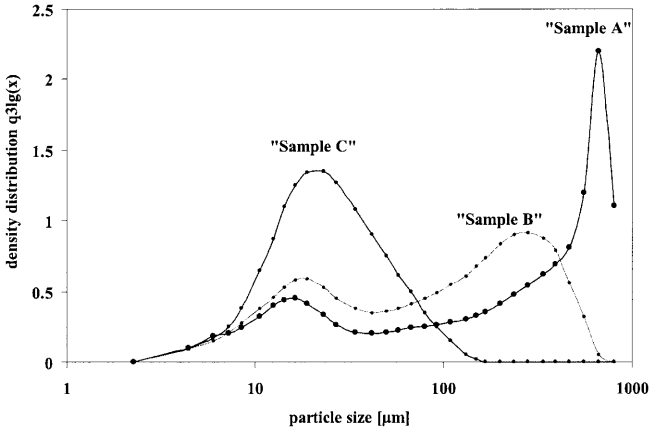

Fig. 2 Particle size distribution of ground maize samples. Sample A was prepared by grinding a maize kernel with a simple (coffee-grinder type) laboratory grinder. Sample B was prepared by grinding a maize kernel in a track mill. Sample C was the final product in the certified reference material production; the maize kernels were ground by a repeated cryogrinding technique

Sample C was the final product in the CRM production; the maize kernels were ground by a repeated cryogrinding technique.

The particle size distribution of these samples is presented in Fig. 2. Samples A and B show two peaks in the distribution. The maximum particle size is greater than 600 and 1000 μm for samples A and B, respectively. Sample C has a much better particle size distribution, every particle is smaller than 150 μm and the maximum of the curve is at 30–40 μm. The calculated number of particles in a 1.00 g sample is 5.07×10^8, 8.21×10^8 and 9.57×10^8 for samples A, B and C, respectively.

The third generation of GMO reference materials is prepared by a dry mixing technique of ground non-GMO with GMO maize kernels. The GMO contents of the mixtures are 0, 0.1, 0.5, 1.0, 2.0, 5.0 and 100%, respectively.

The computer program developed can take the effect of dilution into consideration and gives an estimation of the theoretical uncertainty of each mixture and grinding method (comparison of samples A–C). The program does not calculate the inhomogeneity within the particle size fraction and other sources of uncertainty; therefore, the real uncertainty is underestimated. For this reason the calculated values can be used for the comparison of differently ground and prepared samples. It supports the decision in the choosing and validation of the preparation method in the production of GMO CRMs.

Results and discussion

The DNA concentration is expressed in units of micrograms per litre, considering that 50 μg/L equals 1 absorbance unit. The extracted DNA concentration was significantly higher in the smaller particle size fraction (Fig. 3).

Considering these results an important question has to be answered: is this difference caused by the different composition of particle size fractions or merely by the efficiency of extraction? To answer this question the 355–500-μm particle size fraction of sample B was ground further, and an extraction of the original and the further ground sample was made. The concentration was 106 ± 19 and 87 ± 3 μg/mL for the original and the further ground samples, respectively.

No significant difference in the DNA content was found; therefore, the particle size below 500 μm does not affect the efficiency of extraction – it affected only the reproducibility of extraction. Consequently, the observed difference in the DNA concentration must have been caused by the different composition of the particle size fractions of the sample. Similar patterns were observed for the Fe, Mn, Mg, Zn, P and K concentrations in the dif-

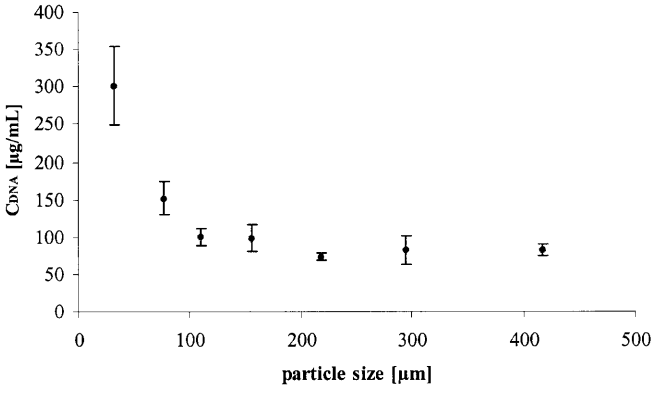

Fig. 3 Concentration of DNA in the different particle size fractions of milled maize kernel sample. Each particle size fraction data represents a range of particle size

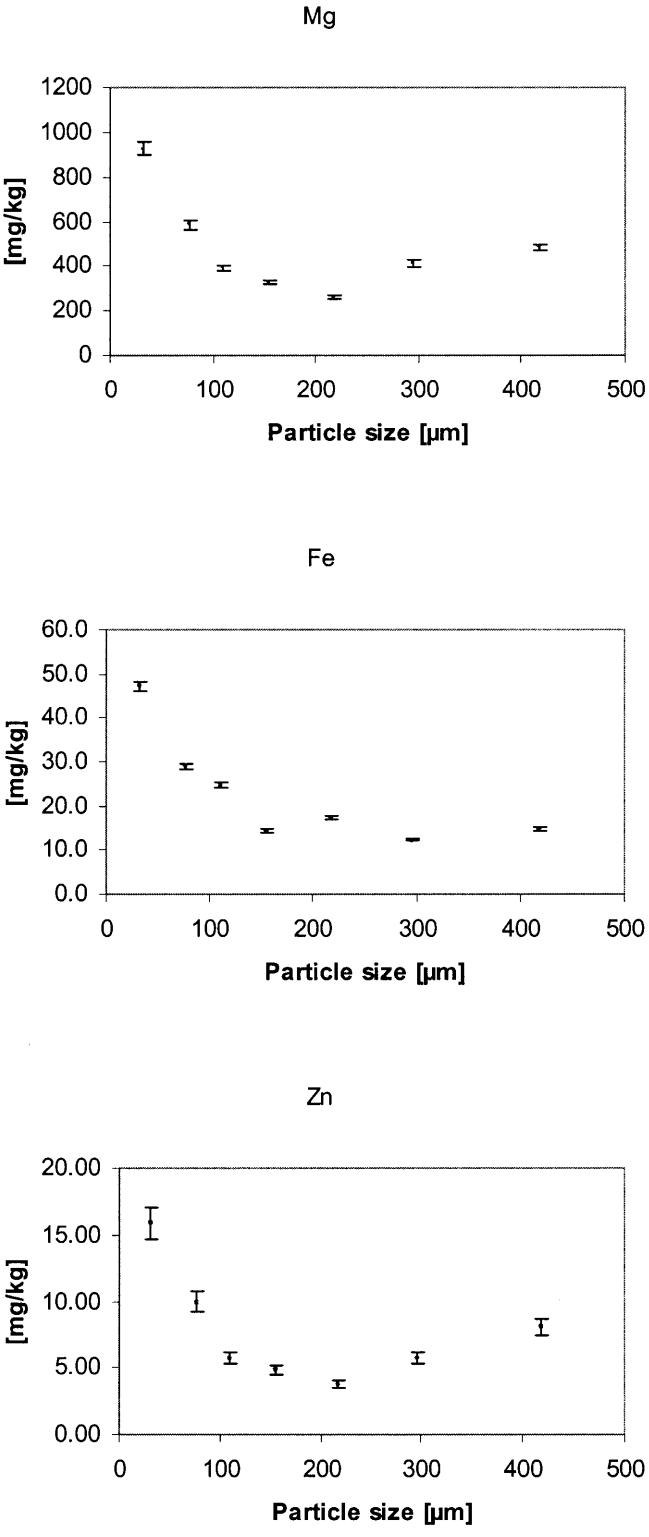

Fig. 4 Concentration of Mn, Fe and Zn in the different particle size fraction of a milled maize kernel sample

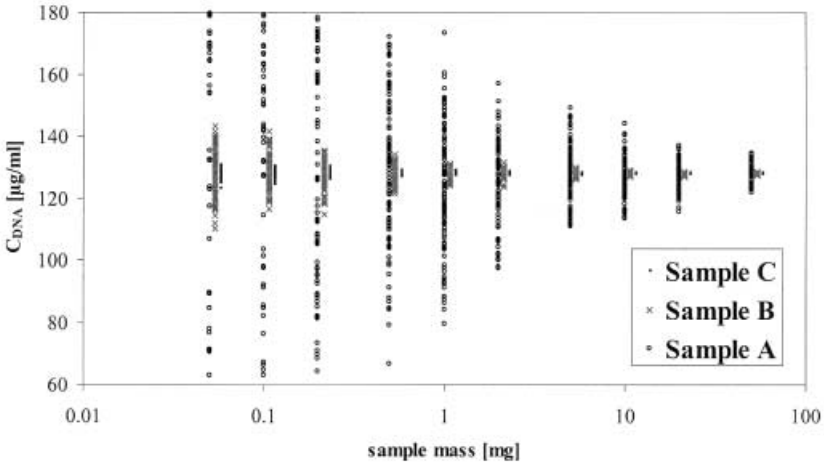

Fig. 5 Repeated running of the simulation program for samples A, B and C. The number of replications was 100 for each sample mass value

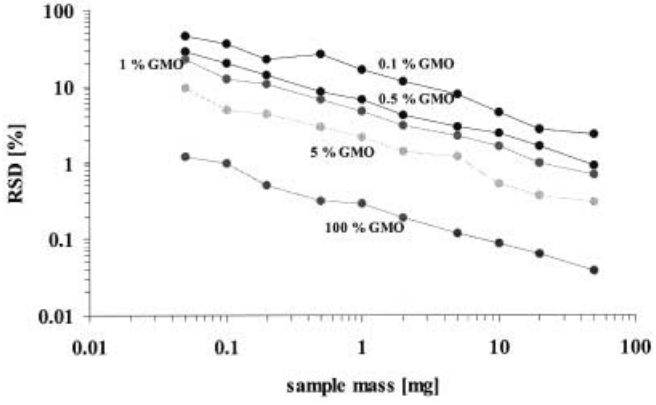

Fig. 6 Calculated relative standard deviation (*RSD*) related to sample mass for sample C. Both scales are logarithmic

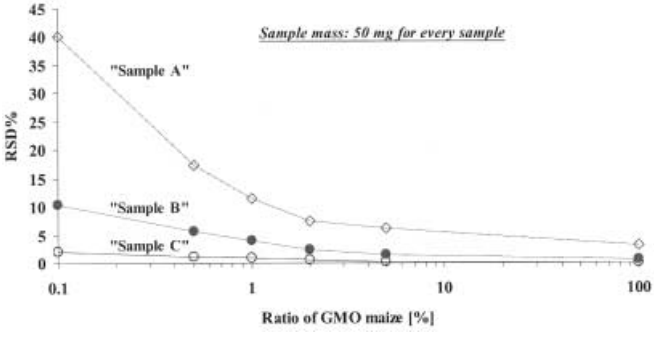

Fig. 7 Effect of solid phase dilution of genetically modified (*GMO*) maize on the RSD of differently prepared maize samples

ferent fractions of milled kernels, which were measured by inductively coupled plasma atomic emission spectrometry, as presented in Fig. 4,.

The results of repeated running of the program for the three samples (A–C) with different sample masses are shown in Fig. 5. The result of the calculation matches the expectations: the deviation of the DNA content is higher for lower sample mass. The program is able to calculate the DNA content in mixed GMO and non-GMO samples as well. The calculated relative standard deviations related to the sample mass are presented in Fig. 6 for different GMO maize concentrations of the final product. At a sample mass of 50 mg, the calculated standard deviations in the DNA content were 0.55, 1.91 and 2.97% if the content of GMO maize in the GMO and non-GMO maize mixtures is 2.0, 0.1 and 0.01, respectively.

The effect of mixing of GMO and non-GMO maize on the standard deviation of DNA content in samples A–C was compared as well. A significant difference in the standard deviation of the samples (Fig. 7) was found. The theoretically minimum standard deviation for a ground maize kernel sample could be 10–20 times higher if the sample is not ground finely enough. The preparation of reference materials from maize kernels with certified GMO DNA content requires very sophisticated production technology. Unsuitable grinding can cause very high relative standard deviation of the measurements and can foil the certification.

In the ground maize kernel sample the DNA concentration is higher in the small particle size fractions. An opposite distribution of analyte could cause a much higher relative standard deviation. By making a calculation for an imaginary sample which has the same particle size distribution as sample A and an opposite distribution of analyte in the particle size fraction more than 150% relative standard deviation can be obtained if the sample amount is 50 mg and the mixing ratio is 1:1000.

The model developed could be a tool to supply consumers and producers with more information related to the quality of CRMs. For the determination of the standard deviation, the sample mass function, the particle size distribution and the concentration distribution have to be determined and the computer program can calculate the function.

The concentration of analyte in the different particle size fractions is often different in biological samples. This type of micro inhomogeneity is not determined during classical homogeneity tests, as described in ISO Guide 31 [7].

The method developed supplies the theoretical minimum for the standard deviation at a certain sample amount.

This type of micro inhomogeneity cannot be determined on the basis of a traditional homogeneity test; the measurement of analyte in the different particle size fractions is required for this.

Application of this numerical method is of great importance for every powdered reference material if the analytical technique applied uses sample masses below 100 mg.

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