

Sampling for Genetically Modified Organisms Content Analysis in Agricultural Products: From Analytical Sample to Test Portion

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Abstract: Objective: At present, Sampling standards and regulations for genetically modified organisms (GMO) are commonly based on theoretical calculations or computer simulations, and there is a lack of field data to validate these simulations. In view of this situation, we sampled agricultural products for GMO content analysis, and investigated the influence of various factors on the accuracy of the results. We have prepared a three-part series and in this part focused on the process from analytical sample to test portions. Method: Using non-transgenic maize as matrix, 12 lines of transgenic maize were used to produce standard analytical samples. After systematic sampling, the GMO contents of these samples were randomly tested, and their single relative standard deviations (RSD) were calculated as a measure of total RSD (single analysis) per sample. Results: By comparing the RSDs of various sampling methods, it was found that the results of 12 strains were basically consistent, and the data of MON810 were listed as a representative. The parameters affecting the standard deviation included the content (a_{AS}), particle size (d_{AS}), test portion mass (M_{TP}) and the number of increments (n_{IT}). Total analytical RSD could be reduced by decreasing particle size, and increasing test portion mass or the number of increments. Based on current laboratory testing conditions and current used kits, for high content analytical sample (>0.01%), more than 2 duplicate test portions with at least -100mesh particle size and 200mg mass were recommended. Conclusion: Based on the results, the recommended values of particle size, test portion mass and the number of increments for the process from analytical sample to test portions were given. These factors were independent on species or strains of the product, so the results were suitable to all species and strains, provided that the solid particles could be crushed to required particle size.

Keywords: Genetically Modified Organisms (GMOs), Sampling, Agricultural Products

1. Introduction

In the past 30 years, the planting area and yield of genetically modified organisms (GMOs) have continuously increased^[1], but its impact on human health is still unclear. Some countries believe that genetically modified products (GM products) are harmless, and GMO lines can be planted and processed provided that they are approved by the government. However, many countries are cautious about the effects of GM products, and have adopted strict technical measures for the cultivation, entry and sale of GM products.

These restrictions have led to frequent disputes in the international trade of GM products, thus raising the requirements for GM testing [2-7].

The way in which the sample is collected plays a vital role in the accuracy of the test results. Accurate sampling of agricultural products containing GM ingredients has become an important research topic for scientists and technicians [8], and the European Union (EU) [9-12], ISO [13, 14] and China [15] have formulated regulations for GMO sampling. However, these regulations rely on theoretical calculations and computer simulations, and analysis using actual data is

lacking.

Several researches on the GM sampling method have been reported [16-22]. Begg *et al* [17] found that under the controlled conditions of a single laboratory, the error associated with the real-time PCR assay to be negligible in comparison with sampling error. Brera *et al* [18] reported the sampling procedures that used 10 increment samples provided the best results, in terms of precision and accuracy. Minkkinen *et al* [20] found that 42 was the absolute minimum number of increments needed for reliable characterization of all lots. Most researches gave summarized suggestion on the whole sampling method, and there is a lack of detailed data on the separated procedure of GM sampling.

GM products are often sampled in three steps: the lot to laboratory sample, the laboratory sample to analytical sample, and the analytical sample to test portion. We will make a detailed study on the whole process in a three-part series, and in this part we have focused on the process of the analytical sample to test portion. Field data has been collected by sampling agricultural products for GMO content analysis, and the influence of various factors on the accuracy of the results have been analyzed; finally we gave the recommended values of particle size, test portion mass and the number of increments for the process from analytical sample to test portions, providing a reference for future studies.

2. Materials and Methods

2.1. Testing Materials

GM reference materials T25, MON88017 and MON87640 were purchased from American Oil Chemists' Society, USA.

GM reference materials TC1507, NK603, MON863, MON810, GA21, BT176, BT11, ES3272 and MIR604 were purchased from Sigma Aldrich (China).

2.2. Instruments

DNA extraction kit for GMO detection Ver2.0 (No. D9093) was purchased from Takara Biomedical Technology (Beijing) Co., Ltd., China.

Real time PCR analyzer (ABI7500) was purchased from Thermo Fisher Scientific Applied Biosystems, USA.

2.3. Preparation of Standard Analytical Samples

After lyophilization, transgenic and non-transgenic maize grains were crushed to -60, -100 and -200 meshes. Using non-transgenic maize as the matrix, the pure maize lines (grain) MON810, Bt11, Bt176, T25, GA21, MON863, NK603, TC1507, ES3272, MON88017, MIR604 and MON87640 were added by the direct weighing method. The addition content of each standard analytical sample was as follows: 10^{-1} ~ 10^{-6} were added in each line (i.e. the mass ratio of 10%~0.0001%), where four lines of analytical samples (MON810, Bt11, Bt176 and T25) with the content of 10^{-1} were added. The V mixer was used to mix samples and to prevent cross contamination. Table 1 shows the typical addition content of the MON810 line.

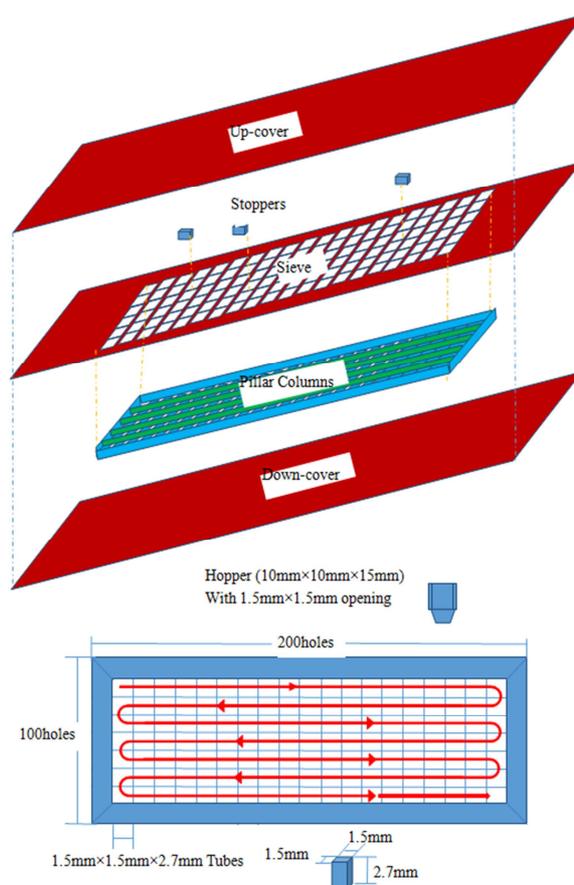


Figure 1. Structure of sampling device.

Table 1. Typical Reference Analytical Sample.

Lines	Addition content	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
	Gross mass, g	100	100	100	100	100	250
M1: maize, -60 mesh	GMO(MON810) Mass, g	10.134	1.012	0.0987	0.0122	0.00109	0.00011
	Matrix mass, g	90.389	99.221	99.856	99.925	100.721	100.454
	Actual content, %	10.08	1.010	0.0987	0.0122	0.00108	0.00011
M2: maize, -100 mesh	GMO(MON810) Mass, g	10.295	1.366	0.1067	0.0091	0.00102	0.00012
	Matrix mass, g	91.478	99.254	99.528	99.473	100.695	100.268
	Actual content, %	10.12	1.358	0.107	0.0091	0.00101	0.00012
M3: maize, -200 mesh	GMO(MON810) Mass, g	10.658	1.201	0.1066	0.0102	0.00117	0.0001
	Matrix mass, g	91.141	99.959	99.791	99.936	100.511	100.104
	Actual content, %	10.47	1.187	0.107	0.0102	0.00116	0.00010

2.4. Test Portion Sampling Device

A minitype sampling device was designed and manufactured; the structure is shown in Figure 1.

2.5. Methods

According to the charging method shown in Figure 1, the reference analytical sample was paved into the microtubes (1.5mm×1.5mm×2.7mm), each micro tube contains about 5mg sample.

For each reference analytical sample, 10 duplicate sets of increments were taken by using systematic sampling method. The mass and number of increments in each duplicate are listed here: 5mg×5, 25mg×1, 5mg×10, 10mg×5, 25mg×2, 50mg×1, 5mg×20, 10mg×10, 20mg×5, 50mg×2, 100mg×1, 10mg×20, 20mg×10, 40mg×5, 100mg×2, 200mg×1.

A 10mg test portion includes samples in two consecutive microtubes, and so on. Combine the selected increments to make up test portions: 25mg×20; 50mg×40; 100mg×50; 200mg×50.

3. Results and Discussion

3.1. Testing Results

GMO contents in the test portions were tested in a random order by the same DNA extraction kit and the same PCR, one result was obtained on one test portion. By comparing the RSDs of various sampling methods, it was found that the results of all strains were basically consistent. The data of MON810 were listed as a representative in table 2, 3 and 4.

Table 2. RSD of test portions from the Reference Analytical Sample and by analysis-M1.

M1: -60 mesh, 25 mg						
Increments	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
5	18.3%	19.4%	20.9%	65.8%	416.8%	931.6%
1	24.7%	28.1%	29.5%	104.0%	422.7%	931.6%
M1: -60 mesh, 50 mg						
Increments	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
10	13.0%	14.8%	12.2%	51.1%	159.9%	785.8%
5	16.7%	18.6%	17.6%	62.7%	393.5%	931.6%
2	18.8%	21.5%	20.9%	76.6%	314.0%	overflow
1	19.8%	19.7%	18.6%	77.4%	262.3%	852.7%
M1: -60 mesh, 100 mg						
Increments	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
20	10.2%	11.9%	9.6%	35.0%	149.4%	598.2%
10	13.2%	13.4%	15.6%	46.2%	244.2%	784.3%
5	13.3%	12.1%	14.4%	47.5%	164.8%	794.9%
2	13.5%	13.5%	13.2%	44.6%	194.6%	854.1%
1	13.7%	15.6%	15.1%	50.2%	317.7%	931.6%
M1: -60 mesh, 200 mg						
Increments	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
20	8.9%	8.2%	8.1%	31.1%	127.0%	574.4%
10	10.3%	9.1%	7.8%	26.7%	125.2%	637.0%
5	10.3%	10.2%	10.4%	33.8%	137.7%	575.6%
2	8.8%	9.8%	10.0%	36.4%	151.9%	779.3%
1	10.1%	10.0%	9.2%	37.0%	144.1%	794.9%

Table 3. RSD of test portions from Reference Analytical Sample and by analysis-M2.

M2: -100 mesh, 25 mg						
Increments	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
5	21.4%	19.7%	20.7%	27.3%	129.2%	784.3%
1	24.7%	27.4%	27.4%	39.8%	185.0%	570.1%
M2: -100 mesh, 50 mg						
Increments	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
10	13.3%	14.6%	13.6%	21.0%	85.2%	413.7%
5	14.2%	17.9%	16.1%	24.2%	122.9%	476.5%
2	16.3%	19.9%	19.4%	25.0%	101.3%	726.5%
1	19.1%	19.1%	20.4%	30.0%	139.9%	716.0%
M2: -100 mesh, 100 mg						
Increments	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
20	10.9%	8.7%	11.1%	16.9%	56.5%	206.3%
10	15.2%	12.9%	12.7%	17.6%	78.4%	243.5%
5	13.4%	13.0%	13.0%	18.7%	75.5%	477.3%
2	14.6%	13.5%	13.8%	18.7%	90.7%	407.4%
1	13.2%	13.6%	13.9%	16.5%	87.2%	398.5%
M2: -100 mesh, 200 mg						
Increments	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶

20	7.2%	9.1%	9.6%	12.8%	49.8%	174.1%
10	9.7%	9.8%	9.4%	14.7%	59.2%	345.7%
5	9.5%	7.8%	10.7%	13.6%	59.9%	258.1%
2	9.5%	8.6%	11.3%	14.9%	69.3%	245.1%
1	9.3%	9.5%	10.1%	13.5%	80.2%	381.4%

Table 4. RSD of test portions from Reference Analytical Sample and by analysis-M3.

M3: -200 mesh, 25 mg						
Increments	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
5	19.8%	18.5%	21.4%	21.6%	41.1%	204.6%
1	23.3%	27.8%	31.2%	29.8%	54.3%	444.8%
M3: -200 mesh, 50 mg						
Increments	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
10	16.3%	14.8%	15.1%	14.5%	30.8%	153.1%
5	18.0%	17.7%	17.2%	16.8%	29.7%	190.4%
2	20.5%	17.9%	17.9%	17.6%	42.3%	279.8%
1	17.3%	20.0%	16.4%	23.3%	35.1%	373.1%
M3: -200 mesh, 100 mg						
Increments	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
20	9.9%	9.1%	10.8%	10.0%	18.7%	120.5%
10	13.0%	13.3%	13.1%	13.3%	27.3%	119.1%
5	11.4%	13.1%	12.8%	15.0%	23.7%	177.8%
2	13.0%	12.1%	13.2%	13.9%	23.4%	132.0%
1	15.4%	17.5%	14.0%	13.3%	26.2%	112.6%
M3: -200 mesh, 200 mg						
Increments	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
20	8.2%	8.6%	9.4%	9.6%	16.7%	93.9%
10	9.9%	8.8%	9.2%	9.4%	18.1%	103.2%
5	9.4%	9.6%	8.7%	10.8%	16.3%	119.4%
2	8.9%	8.7%	10.3%	10.3%	18.6%	101.0%
1	9.3%	9.5%	10.3%	10.6%	18.8%	106.6%

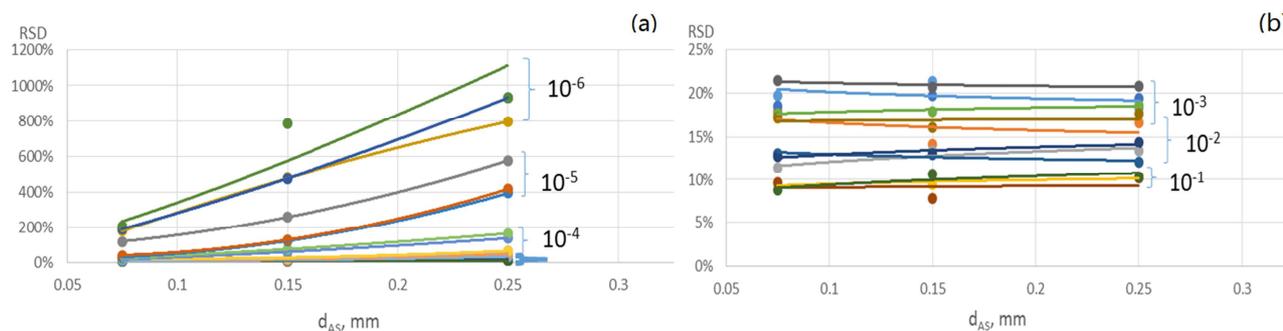
3.2. Analysis and Optimization of Parameters

3.2.1. Parameters Affecting RSD

According to Table 2-4, the main parameters influencing the RSD of analytical samples included the content a_{AS} (AS, Analytical Sample), particle size d_{AS} , test portion mass M_{TP} (TP, Test Portion), the number of increments n_{IT} (IT, Increase composition of Test Portion).

3.2.2. Optimization on Particle Size of Analytical Samples

The nominal (particle size) of analytical sample is of great concern to RSD, as shown in Table 2-4. The overall trend is that smaller particle size leads to smaller RSD. On the higher level (10^{-1} ~ 10^{-3}), particle size has little effect on RSD, this may be explained by increasing aggregated distribution; on the lower level (10^{-4} ~ 10^{-6}), the effect of particle size to RSD increase with the content level going down (see Figure 2).



(a) RSD between 0% and 1200%, (b) Enlarge the RSD part of (a) between 0% and 25%.

Figure 2. Effect of particle size of analytical sample on RSD.

Theoretically, solid sample could be crushed to a very fine size (e.g. -10000mesh), however in practice, crushing the laboratory sample unboundedly will waste time and money, even impossible and unnecessary. For agricultural product with high oil content, it is impossible to be crushed to -200mesh, because in this case the product has become thick

or their property has been changed due to high temperature. For the products studied, it is feasible to crush them to -100mesh. For the routine sampling and analysis, the analytical sample is suggested to be crushed to -100mesh or lower.

For high content analytical samples (10^{-1} ~ 10^{-3}), there is no

significant difference on RSD between -100mesh and -200mesh samples, when the mass of test portion is over 100mg, they are also almost the same for low content (10^{-4}), however, the RSD difference will be 4~5 times for low content samples ($10^{-5}\sim 10^{-6}$). That's to say, for the analytical samples with over 10^{-4} content, reducing particle size is unnecessary, only when the content is lower than 10^{-4} , it is economical for reducing the particle size. On the other hand, RSD could also be reduced by increasing the mass of test portion or the number of increments, reducing particle size is not always the best and necessary option.

In order to keep the consistency with routine analytical conditions, the particle size of the analytical sample is fixed to -100mesh hereafter. On the study stage, some analytical samples were crushed to -200mesh after lyophilization to prevent over estimation of the total analytical variance.

3.2.3. Minimum Mass of Test Portion

The effect on total analytical RSD by different mass of test portion is shown in Figure 3. In the range given in current analytical methods (25~300mg), RSD tends to be lower down with increasing mass of test portion. When it is over 100mg, the falling rage becomes narrow. RSDs for 100mg and 200mg test portions only differ 1.5 times, and those for 50mg and 100mg differ 2.3 times. Analysing the trend of data, the reasonable mass is 200mg.

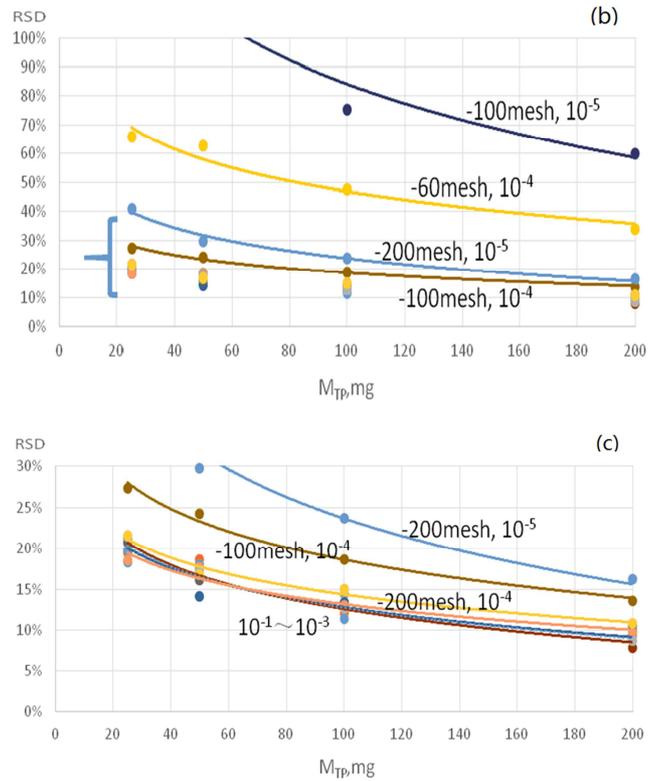
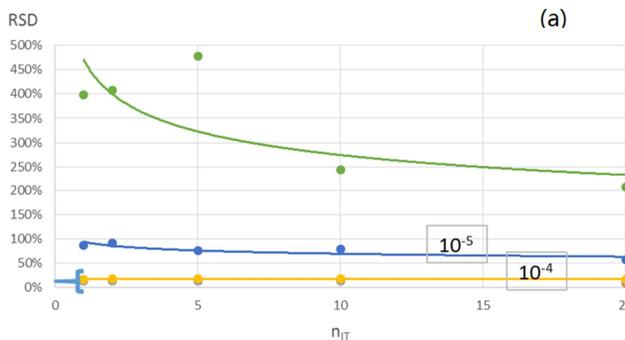
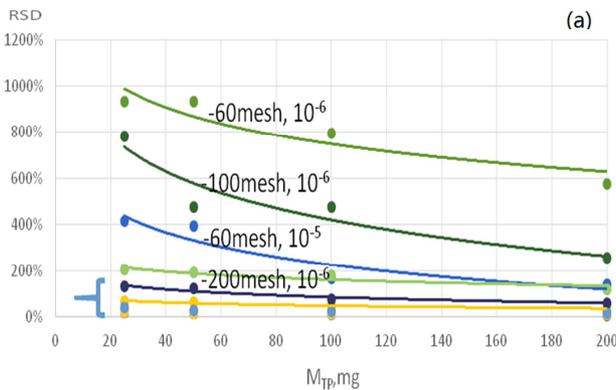


Figure 3. Trends of RSD against mass of test portion.

(a) RSD between 0% and 1200%, (b) Enlarge the RSD part of (a) between 0% and 100%, (c) Enlarge the RSD part of (b) between 0% and 30%.

3.2.4. Number of Increments Composing of Test Portion

For -100mesh 100mg test portion, plot RSD against n_{IT} in Figure 4. It shows that the effect of n_{IT} on RSD is obvious only when the content is very low ($10^{-5}\sim 10^{-6}$). More increments ($n_{IT} \geq 5$) are recommended to be taken to make up a test portion, because this operation is very easy, taking test portion one-off should be avoided, so that the distribution variance of the analytical sample could be reduced as far as possible.



(a) RSD between 0% and 500%, (b) Enlarge the RSD part of (a) between 0% and 30%.

Figure 4. Influence of the number of increments on RSD.

3.3. Analysis of RSD Derived from Various Approaches of Sampling and Testing

The total analytical RSDs of the results from the analytical

samples by different sampling and assay approached were evaluated and listed in Table 5, where the particle sizes were -100mesh and -200mesh, the masses of test portions were 50 to 200mg, 5 increments were taken to make up a test portion,

single extraction of DNA and single PCR analysis for each of 1 to 3 duplicate test portions were carried out to obtain GMO contents.

In Table 5, in the range of high content (10^{-1} ~ 10^{-3}), in order to control the total analytical RSD less than 10%, two duplicate test portion with the mass more than 100mg and particle size lower than -100mesh should be analyzed; if the control limit is 14.1%, the mass of each test portion could be reduced to 50mg. In routine procedure, analyzing two duplicates on -100mesh 100mg test portions is feasible; it

means the RSD could be controlled lower than 10%. In the range of medium content (about 10^{-4}), RSD could be limited lower than 14.1%, by using two-200mesh, 100mg duplicate test portions; in the range of low content (about 10^{-5}), the RSD is hardly to be controlled to be lower than 20% when -100mesh 200mg test portions were used, but if three -200mesh 100mg test portions were used; in the very low content (10^{-6} or lower), even -200mesh analytical sample were used, the RSD can be hardly controlled less than 50%, for three duplicate, the RSD is still larger than 70%.

Table 5. RSDs by Different Approaches.

Size/MTP	N_{TP}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
-100 mesh, 50mg	1	14.2%	17.9%	16.1%	24.20%	123%	477%
	2	10.0%	12.7%	11.4%	17.1%	87.0%	337%
	3	8.2%	10.3%	9.3%	14.0%	71.0%	275%
-100 mesh, 100mg	1	13.4%	13.0%	13.0%	18.7%	75.5%	477%
	2	9.5%	9.2%	9.2%	13.2%	53.4%	337%
	3	7.7%	7.5%	7.5%	10.8%	43.6%	275%
-100 mesh, 200mg	1	9.5%	7.8%	10.7%	13.6%	59.9%	258%
	2	6.7%	5.5%	7.6%	9.6%	42.4%	182%
	3	5.5%	4.5%	6.2%	7.9%	34.6%	149%
-200 mesh, 50mg	1	18.0%	17.7%	17.2%	16.8%	29.7%	190%
	2	12.7%	12.5%	12.2%	11.9%	21.0%	134%
	3	10.4%	10.2%	9.9%	9.7%	17.1%	110%
-200 mesh, 100mg	1	11.4%	13.1%	12.8%	15.0%	23.7%	178%
	2	8.1%	9.3%	9.1%	10.6%	16.8%	126%
	3	6.6%	7.6%	7.4%	8.7%	13.7%	103%
-200 mesh, 200mg	1	9.4%	9.6%	8.7%	10.8%	16.3%	119%
	2	6.6%	6.8%	6.2%	7.6%	11.5%	84%
	3	5.4%	5.5%	5.0%	6.2%	9.4%	69%

Because the commonly used PCR reaction systems require DNA concentration 50~100ng/iL and the volume of DNA extraction solution 25~100iL, that is, DNA mass in the solution is 1~10mg, 50mg test portion is suitable for about 10^{-2} GMO content; 100mg and 200mg are suitable for about 10^{-3} ; and when 10^{-4} content level is tested, more test portion or further concentrating of DNA extraction solution should be used, so that the concentration of DNA could fall in 50~100ng/iL (while the quantitative limit is 1ng/iL, equivalent to 100 copies in 2iL). In practice, using 10iL DNA extraction solution, the concentration of the solution extracted from 100mg test portion with 10^{-4} content is about 1ng/iL, and for 10^{-5} , the mass of test portion needs to be 1g, and for 10^{-6} , 10g. This is the reason why the total analytical

RSD could not be reduced for low and very low content samples.

50~300mg test portion is used by current DNA extraction kits, according to above discussion, the kits with this range of mass could only be used for the samples which GMO content higher than 10^{-4} . Therefore, based on current level of PCR test, the limit in regulation should not be set lower than 10^{-4} . Due to the same consideration, in this project, we will focus on those with GMO content higher than 10^{-5} , and the limit of RSD(TP) is set on 10%. The very low content sample ($\sim 10^{-6}$) will not be studied.

According to the above discussion, recommended approaches for taking test portions from the analytical sample and expected RSD are listed in Table 6.

Table 6. Recommended sampling approaches for routine analysis.

Controlled RSD	Content a_{AS}	Particle Size	M_{TP} , mg	n_{TP}
~10%	$\geq 0.1\%$	-100 mesh	100	2
%	$\geq 0.01\%$	-100 mesh	100	3

4. Conclusions

The relative sampling RSD (TP) mainly depends on the particle size of the analytical sample and the mass test portion, i.e. the inherent heterogeneity of the analytical sample plays a key role. The number of increments constituted the test portion affect RSD (TP) to some extent. The main approaches for reducing the total analytical RSD (TAE) are reducing particle

size of analytical sample, increasing mass of test portion and increasing duplicate number.

In practice, based on current laboratory testing conditions and current used kits, for high content analytical sample ($>0.01\%$), it is necessary to use more than 2 duplicate test portions with at least -100mesh particle size and 200mg mass. With the current analytical techniques, it is meaningless to study or analyze very low content samples (such as 10^{-6}).

For the analytical sample which is fit for the purpose of

analysis, the number of increments constituting the test portion has weak effect on RSD(TAE), however, taking test portion one-off from the analytical sample is not recommended, the number prefers to be about 5.

The conclusions in this article are based on the content, particle size of analytical sample, the number of increments constituting test portion and mass of test portion. These factors are independent on species or strains of the product, so the conclusions are suitable to all species and strains, provided that the solid particles could be crushed to required particle size.

Acknowledgements

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