

JRC TECHNICAL REPORT



Guidelines for sample preparation procedures in GMO analysis

Prepared by the ENGL ad hoc working group on “sample preparation procedures”

2014

Report EUR 27021 EN

European Commission

Joint Research Centre
Institute for Health and Consumer Protection

Contact information

Molecular Biology and Genomics Unit
Address: Joint Research Centre, Via Enrico Fermi 2749, TP 201, 21027 Ispra (VA), Italy
E-mail: eurl-gmff@jrc.ec.europa.eu
Tel.: +39 0332 78 5165

JRC Science Hub

<https://ec.europa.eu/jrc>

Legal Notice

This publication is a Technical Report by the Joint Research Centre, the European Commission's in-house science service. It aims to provide evidence-based scientific support to the European policy-making process. The scientific output expressed does not imply a policy position of the European Commission. Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use which might be made of this publication.

All images © European Union 2014

JRC94042

EUR 27021 EN

ISBN 978-92-79-44704-4 (PDF)

ISBN 978-92-79-44705-1 (print)

ISSN 1831-9424 (online)

ISSN 1018-5593 (print)

doi:10.2788/738570

Luxembourg: Publications Office of the European Union, 2014

© European Union, 2014

Reproduction is authorised provided the source is acknowledged.

Abstract

This document aims at providing guidelines for a correct sample preparation in GMO analysis on food, feed, seed, plants and propagating material samples. It has been developed by the European Network of GMO Laboratories (ENGL), specifically by the ENGL working group "sample preparation procedures" upon a mandate of the ENGL steering committee. The structure of the document is based on that of an existing ISO standard (ISO 6498:2012), dealing with sample preparation for feed; starting from this, the procedures were adapted to the needs of GMO detection and the scope concerning matrices was widened to food, feed and seeds.

These guidelines cover the key steps of the sample preparation, i.e. the size of the laboratory sample, the mass reduction techniques as well as techniques for comminution and mixing; considerations about the test portion are included and some performance tests to be applied at different steps of the process are provided.

While sample preparation, falling after sampling and before analytical testing for GMOs, has suffered from a lack of guidance and harmonization, this document provides detailed and practical hints on this important step, thus contributing to foster testing harmonisation.

Executive summary

This document has been developed by the Members of the working group 'sample preparation procedures' based on a mandate of the ENGL steering committee that is detailed in the introduction. The general structure of the document is based on that of an existing ISO standard (ISO 6498:2012) handling specifically about sample preparation for feed. Most of the definitions are taken from this standard. The procedures were however adapted for GMO detection and the scope concerning matrices is much wider (food, feed and seeds). This has an impact for instance on mass reduction processes because risk of contamination is of more concern in GMO detection. There is a brief reminder of the theory of sampling. Within the procedure the most important points are: the size of the laboratory sample, the mass reduction techniques as well as techniques for comminution and for mixing and finally considerations about the test portion. Some performance tests to be applied at different steps of the process are provided. As a conclusion it is assessed in how far the expectations of the mandate were addressed, gaps were identified and proposals are made for the future.

Content

Executive summary i

Content.....ii

Introduction..... 1

1 Scope 2

2 Principle 2

3 Definitions 2

4 General considerations related to the theory of sampling 4

5 Equipment 8

6 Environment and rooms..... 10

7 Safety precautions..... 10

8 Procedure 11

9 Performance tests (quality control)..... 22

10 Identified gaps and conclusions 24

11 Bibliographic references..... 26

Annex I..... 29

Annex II..... 30

Annex III..... 31

Annex IV 32

Annex V..... 33

Annex VI 35

Annex VII 36

Introduction

The ad hoc working group on sample preparation procedures was established on the basis of a mandate adopted at the 19th ENGL steering committee meeting of 6th of October 2010. The working group has been chaired by Gilbert Berben, Walloon Agricultural Research Centre (CRA-W), Gembloux, Belgium. The other members of the working group have been: Diana Charels, Joint Research Centre (JRC), Institute of Health and Consumer Protection (IHCP), Ispra, Italy; Tina Demšar, National Institute of Biology (NIB), Ljubljana, Slovenia; Rupert Hohegger, Austrian Agency for Health and Food Safety (AGES), Vienna, Austria; Elena Nardini, Joint Research Centre (JRC), Institute of Health and Consumer Protection (IHCP), Ispra, Italy; Roberta Onori, Istituto Superiore di Sanità (ISS), Rome, Italy; Manuela Schulze, Rötgesbüttel, Germany; Patrick Philipp, Service Commun des Laboratoires, Strasbourg, France; Thomas Weber, Joint Research Centre (JRC), Institute of Health and Consumer Protection (IHCP), Ispra, Italy.

The mandate of the working group was the following:

- *Evaluation of the reference documents (ISO norms, CEN standards, legislative provisions) at national, European and international level related to sample preparation issues, both in general and focused on GM product analysis.*
- *Characterization of the most relevant scientific publications associated with sample preparation issues.*

The text of the mandate also stressed particular issues to consider within the working group:

Within the cited reference documents and publications the following issues will be covered:

- *Evaluation of the correlation between particle size and DNA extraction yield for GMO crops.*
- *Comparison and evaluation of pros and cons derived from the use of different grinding devices.*
- *Evaluation of cross contamination problems.*
- *Comparison between slurry technique versus dry milling.*
- *Evaluation of practicability in terms of time of grinding operations, safety of operators and of working areas environmental issues.*
- *Evaluation of the weight of each of the above considered issues in order to analyze their contribution and identify the best sample preparation procedure.*
- *Set up of ad hoc standard operating procedures.*

Finally the mandate also gave some indications about the expected outcome:

All this information should represent the basis to building up an operative guideline for laboratory technicians and to identifying correct pathways for a reliable analysis.

This document is the result of the ENGL working group on sample preparation procedures. However it was based on other existing documents and mainly on an ISO document that existed as a draft when the working group started its mandate:

'Animal feeding stuffs — Guidelines for sample preparation' as document ISO 6498 jointly prepared by Technical Committee ISO/TC 34, Food products, Subcommittee SC 10 and Technical Committee CEN/TC 327, Animal feeding stuffs (ISO/FDIS 6498:2011(E)) It gave rise to the standard ISO 6498:2012 which specifically states that its guidelines are overruled by special instructions and regulations for sample preparation demanded by specific analysis methods.

Since GMO analysis using DNA-based techniques has special requirements related to cross-contamination risks, a specific document was still deemed necessary and the present report kept the format of a standard. The rules or guidelines described in it may probably be applied to any other DNA-based analysis covering the same range of matrices as those considered here for GMOs (food, feed and seeds).

The guidelines were elaborated with the perspective that the test portion would be submitted to a DNA extraction step in view of a PCR analysis. However most of the principles outlined here should remain valid even if the final target analyte does not consist of DNA but of proteins (or polypeptides).

Guidelines for sample preparation procedures in GMO analysis

1 Scope

This document aims at providing guidelines for a correct sample preparation during GMO analysis on food, feed, seed, plants and propagating material samples. For seed sample preparation the reader is also referred to the ISTA rules (ISTA, 2013).

2 Principle

The sample preparation guidelines describe the procedure for handling a laboratory sample upon its arrival in the laboratory to obtain homogeneous and representative test portions avoiding cross-contamination.

In general the whole laboratory sample is homogenised to obtain a test sample for the analysis. Sometimes mass reduction (sub-sampling) has to be done. The procedure followed has to be documented.

All the sample preparation steps should be done under conditions avoiding cross-contaminations and minimising degradation of the target analyte (DNA or proteins) in the test portion.

Storage of the samples prior to and during the sample preparation as well as after the process should be performed under adequate conditions (e.g. at room temperature, refrigerated, frozen) depending on the sample.

3 Definitions

The following section provides an overview of definitions generally taken from GMO European legislation and standards (ISO and CEN).

3.1 General definitions

3.1.1 Lot

A *lot* is a distinct and specified quantity of material dispatched or received at one time and covered by a particular contract or shipping document (Commission Recommendation 787/2004).

3.1.2 Grab sampling

Any 'lump' or material from the lot, taken in one single operation (Minkkinen and Esbensen, 2009). Adapted at laboratory level in the context of sample preparation, it means that this material is taken from the laboratory sample or from the test sample. The focus of grab sampling is exclusively on getting the final subsample mass in one go (based on Petersen et al., 2004). Or in other words: *Catch whatever you can in the cheapest possible way!* (Lischer, 2001a).

3.2 Definitions concerning the 'sample'

Since the model taken for this text is based on the standard for sample preparation relating to animal feeding stuffs, the working group recommends to use the definitions listed in ISO 6498:2012 with a small adaptation of the definition regarding the 'reserve sample':

3.2.1 Laboratory sample

Sample as prepared (from the lot) for sending to the laboratory and intended for inspection or testing (ISO/FDIS 6498:2011).

3.2.2 Reserve sample

Left-over material from the test samples where test portions (or from the laboratory sample if both are the same) are taken away from and where no further particle size reduction is done (adapted from ISO/FDIS 6498:2011).

3.2.3 Test portion

Quantity of material drawn from the test sample (or from the laboratory sample if both are the same) (ISO/FDIS 6498:2011).

NOTE: the definition for 'test portion' provided by ISO 24266:2006 adds aspects that are not considered here: sample, as prepared for testing or analysis, the whole quantity being used for analyte extraction at one time.

3.2.4 Test sample

(Sub-) sample prepared from the laboratory sample and from which test portions will be taken (ISO/FDIS 6498:2011).

NOTE: mind that the definition provided here corresponds to what is called the 'analytical sample' in Commission Recommendation 787/2004 (European Commission, 2004) while the concept of test sample in this recommendation is set equal to the notion of test portion as defined in ISO 24276:2006 and CEN TS 15568:2007.

3.2.5 Other definitions related to the sample

Other definitions of the concept of 'sample' and closely related issues according to various standards and regulatory texts are detailed in **Annex I**.

3.3 Definitions concerning the 'sample preparation procedure'

3.3.1 Coarse grinding (or pre-grinding)

A first grinding step of the whole sample to be carried out before mass reduction when the laboratory sample contains large lumps or if its mean particle size exceeds about 6 mm (ISO/FDIS 6498:2011).

3.3.2 Comminution

Any process (see a non-exhaustive list of possibilities in **8.3.1**) that enables reduction of the average particle size of a solid material and thereby reduces its constitutional heterogeneity.

3.3.3 Constitutional (compositional) heterogeneity

Definition of the concept can be found under paragraph **4.1.1**.

3.3.4 Distributional heterogeneity

Definition of the concept can be found under paragraph **4.1.2**.

3.3.5 Fragment — particle

The smallest physically separable unit that is not affected by the subsampling process (based on Petersen et al., 2005). Comminution will affect the mean size of the fragments or particles.

3.3.6 Homogeneity

Degree to which a property or a constituent is uniformly distributed throughout a quantity of material (ISO/FDIS 6498:2011).

NOTE: Homogeneity may be considered to have been achieved in a practical sense when the sampling error of the processed portion is negligible compared to the total error of the measurement system. Since homogeneity depends on the size of the units under consideration, a mixture of two materials may not be homogeneous at the molecular or atomic level, but homogeneous at the particulate level. However, a uniform visual appearance does not ensure compositional homogeneity (ISO/FDIS 6498:2011). Heterogeneity has a larger impact on errors when the concentration of the property or the constituent to measure is low in the sample.

3.3.7 Increment

An increment in this context will be considered as a portion of material extracted from the laboratory sample when a sub-sampling process is required (adapted from Minkinen and Esbensen, 2009).

3.3.8 Incrementing (composite sampling)

Collection of many random increments from the laboratory sample to make up the subsample. More generally incrementing can be considered as the combination of a number of random increments to obtain a sub-sample before further processing or further analysis (based on ISO/FDIS 6498:2011).

NOTE: To meet the fundamental sampling principle, all increments must have the same probability of ending up in the sample.

3.3.9 Mass reduction

Part of the sample preparation procedure to reduce the mass of a laboratory sample through subsampling (ISO/FDIS 6498:2011).

NOTE: After mass reduction all subsamples should have the same properties as the original laboratory sample (ISO/FDIS 6498:2011).

3.3.10 Mixing-blending-homogenisation

Any process that enables a reduction of the grouping and segregation effects and thereby reduces the distributional heterogeneity of a sample.

NOTE: Comminution processes like grinding may not only contribute to particle size reduction but can also homogenize the sample.

3.3.11 Particle size reduction

Part of the sample preparation procedure done by chopping, crushing, cutting, blending (homogenising), milling (grinding), pulverising to obtain a homogenous test sample for further analysis (based on ISO/FDIS 6498:2011).

NOTE: In general the particle size reduction follows the mass reduction step of the sample preparation procedure with different sieve-size-options to ensure integrity of the test sample(s) (ISO/FDIS 6498:2011).

4 General considerations related to the theory of sampling

Sample preparation steps have been shown to contribute to the largest error component in an analytical determination. This kind of error, which is generally overlooked, is in essence a sampling error and therefore it is generally much larger than the errors arising from subsequent analytical procedures. To fully comprehend the reasons of some actions to be undertaken in sample preparation it is important to understand possible error sources and the means to reduce them.

4.1 Errors linked to characteristics of the material under analysis

Errors deriving from sample heterogeneity (i.e. properties of the material under analysis) contribute to the total subsampling error in two ways: through constitutional heterogeneity and through distributional heterogeneity. One should also bear in mind that the level of the analyte to be measured has a decisive influence on the possible error size. It must therefore be considered as a rule of thumb that the lower the content of the target analyte, the higher the measurement uncertainty on this parameter.

4.1.1 Constitutional heterogeneity

When a great overall composition-based difference exists between the individual smallest inseparable fragments of a sample exists, the constitutional heterogeneity is large (**Figure 1**). The total contribution to heterogeneity is never zero, however, as that would be the case of all fragments being strictly identical. Mixing and blending does not change constitutional heterogeneity.

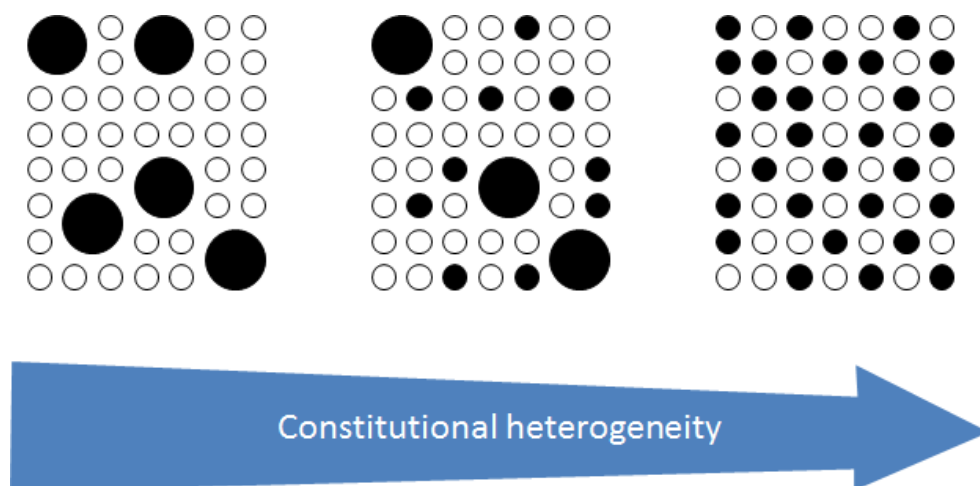


Figure 1 — Illustration of a decreasing constitutional heterogeneity

The only way to alter the constitutional heterogeneity of any given material is by comminution (crushing /cutting) or by other methods changing the physical properties of a sample. The reduction of the average particle size is the dominating factor in reducing constitutional heterogeneity. The other possibility to limit the constitutional heterogeneity is to consider a sufficient mass of test portion that can reflect the properties of the sample. In most cases this is not an option because in GMO detection the test portion size should generally remain small to be compatible with extraction procedures.

4.1.2 Distributional heterogeneity

The grouping and segregation error is linked to the distributional heterogeneity. This heterogeneity reflects the non-random distribution of particles in the sample. It results mainly from the gravitational forces on particles of different densities, sizes and shapes which cause grouping and segregation of all particles. Particles with large differences in size and/or density tend to segregate or stratify heavily, with the smallest and/or densest particles at the bottom of the sample.

Mixing and blending are techniques that can reduce the grouping and segregation error.

Distributional heterogeneity can best be illustrated by a laboratory sample consisting of black and white spheres. If all the black spheres are located at the bottom of the sample and the white spheres are more to the top, the system displays a very high distributional heterogeneity. If on the other hand the spheres have been mixed well (i.e. homogenised), the system's distributional heterogeneity would be significantly reduced (**Figure 2**).

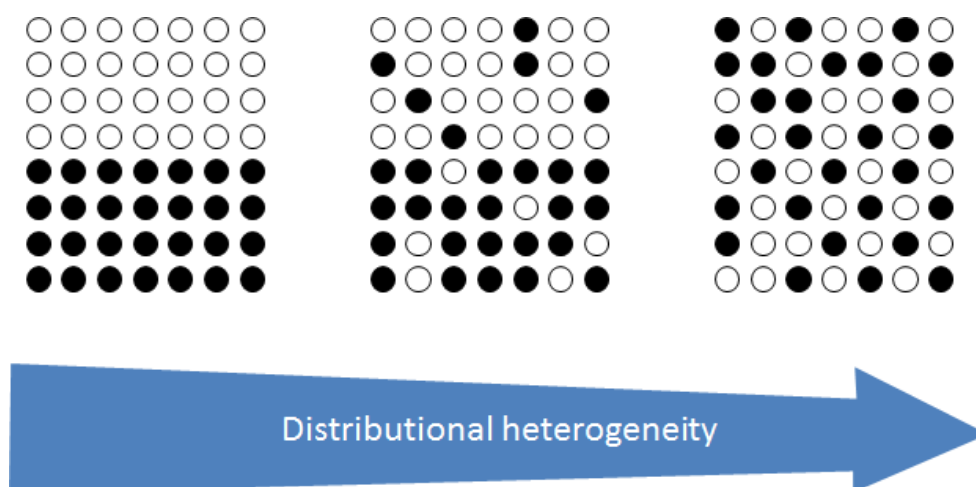


Figure 2 — Illustration of a decreasing distributional heterogeneity

Mixing is not an appropriate technique for many materials because it may, for some materials and under certain circumstances, increase segregation instead of reducing the grouping and segregation error. Segregation will always occur because of the forces of gravity. Many materials will always display an innate propensity for segregation, even immediately after mixing, e.g. highly density-fractionated materials and suspensions. Such systems require constant monitoring and treatment, but once this feature has been duly recognised it can always be dealt with.

Incrementing is another technique which helps to reduce the distributional heterogeneity.

4.2 Errors linked to the subsampling process

The way in which subsampling is achieved is also a source of possible errors. Any subsampling process produces its own sampling errors. Incorrect sampling errors (ISE) are to be avoided at any cost. These errors are the root cause of all sampling bias. Elimination of ISE therefore has absolute priority in all practical subsampling steps. These errors can be subdivided in three main types (Esbensen and Julius, 2010): increment delineation errors, increment extraction errors and increment preparation errors.

4.2.1. Increment delineation errors

Errors linked to a delineation of the increment introduce a bias whereby the distributional heterogeneity of the increment systematically increases or decreases towards the mean distributional heterogeneity of the sample.

In correctly delineated increments any part of the subsample or laboratory sample is represented in identical proportions. For three-dimensional samples it is important to subsample also at the very bottom layer (to avoid the so-called '*Brazil nut effect*' linked to gravity). For material spread in a long pile, a complete cross-sectional slice avoids the increment delineation error. This concept can be illustrated by the use of shovels which are not square-edged (see **Figure 3**). In the illustrated examples, the correct delineation of the increment is shown in B and D where all material in between the vertical lines is sampled. The extraction is done incorrectly in A and C because of the extraction tools (e.g. round-edged shovel) used which results in an incomplete sampling of the material in between the vertical lines (see **4.2.2**).

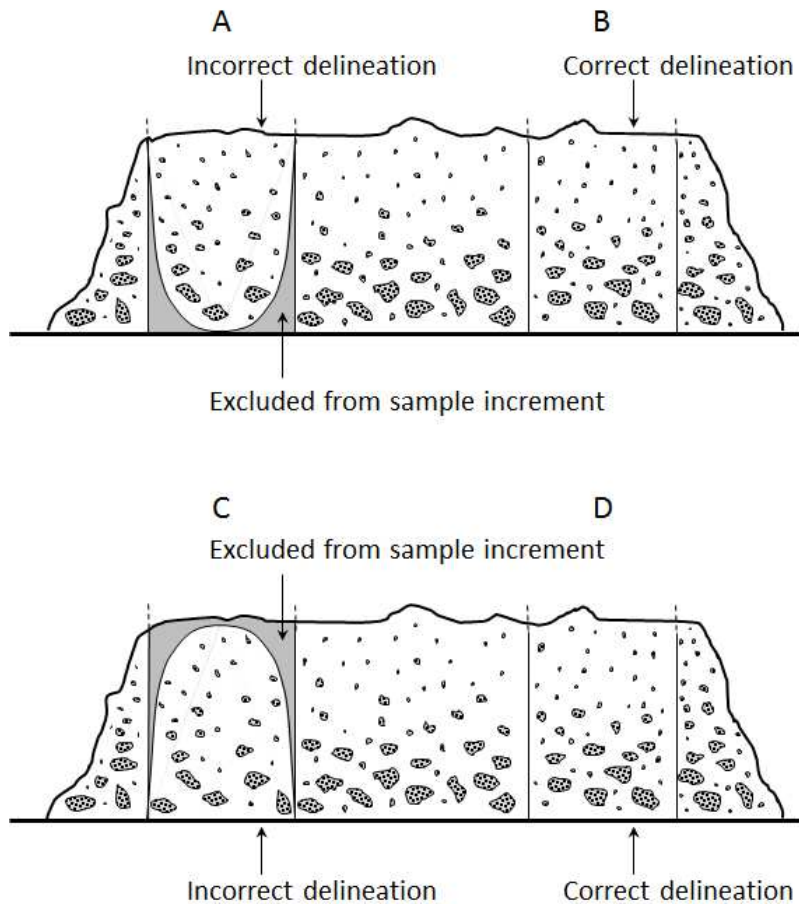


Figure 3 — Use of inappropriate extraction tools can result in an incorrect delineation of the increment that will lead to over-representation (C) or under-representation (A) of some fractions present in the sample (figure adapted from Myers, 2004).

4.2.2 Increment extraction errors

This error arises when fragments inside the delineated increment do not end up in the composite subsample (due to bouncing or dusting or due to the use of inappropriate sampling tools) or if fragments outside the delineated increment migrate into the composite sub-sample. Only fragments with their centre of gravity inside the several delineated increments should become part of the composite subsample (as illustrated in **Figure 4**).

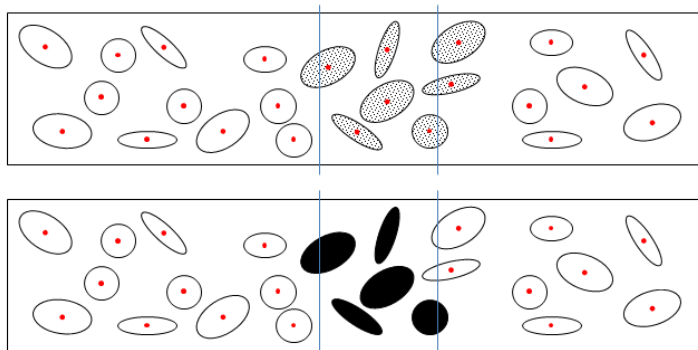


Figure 4 — Sample extraction — only those fragments with their centre of gravity (red dot) within the delineated increment should be extracted. The upper part of the figure shows all fragments which are in the increment or at least overlap it while the lower part illustrates which fragments (in black) have to be extracted (adapted from Esbensen and Paasch-Mortensen, 2010).

4.2.3 Increment preparation errors

This error arises when the sample is altered during its handling (e.g. contamination).

4.3 Subsampling operations

There are only four subsampling operations to reduce the subsampling errors, (Esbensen and Paasch-Mortensen, 2010):

- Composite subsampling (or incrementing),
- Comminution (particle size reduction),
- Mixing/blending,
- Representative mass reduction.

The first three aim to limit errors linked to the characteristics of the matrix under analysis. The last operation is linked to how subsampling itself has to be carried out in order to avoid incorrect subsampling errors.

Representative mass reduction is a way to perform correct subsampling and avoid incorrect subsampling errors (ISE).

5 Equipment

In the section below, examples of equipment which may be needed in sample preparation are given. The selection of the equipment depends on the specific needs of the laboratory.

5.1 Cleaning tools

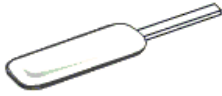
- a) **Brushes** for cleaning grinders, etc.
- b) **Compressed air blower** for cleaning
- c) **Vacuum cleaner**
- d) **Ultrasound bath** (e.g. useful for cleaning sieves)

5.2 Sampling tools

Spoons, spatulas and shovels.

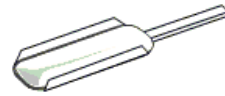
To avoid the increment delineation errors it is advised that spoons, spatulas and shovels should be square-edged (e.g. **Figure 5**).

Incorrect design



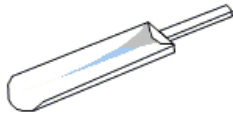
Flat without edges: material segregates when falling off each side

Correct design



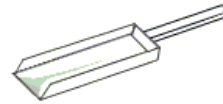
Square edges: prevent material from falling off each side

Spatula



Round shape: material at the top of the flattened sample has more chance to be part of an increment than the material at the bottom

Scoop

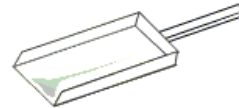


Square shape: all material has the same chance to be part of an increment



Round shape: material at the top of the flattened sample has more chance to be part of an increment than the material at the bottom

Shovel



Square shape: all material has the same chance to be part of an increment

Figure 5 — Some recommendations related to appropriate design of spatulas, scoops and shovels used in sampling (figure adapted from Myers, 2004).

5.3 Sample preparation

5.3.1. Balance

5.3.2. Seed counting device (optional)

5.4 Drying systems

5.4.1 Lyophilisation (or freeze-drying) system

5.4.2 Drying ovens or incubators

5.5 Equipment for particle size reduction and mixing

5.5.1 Mass reduction devices: riffle splitters

5.5.2 Grinders

For practical examples of grinders used in GMO laboratories, see **Annex II**.

5.5.3 Dust extractor

5.5.4 Mortar and pestle or more automatic disruption devices (e.g. TissueLyser)

5.5.5 Overhead mixer, tumbling mixer and other types of mixers (e.g. rotating mixer, ...)

5.5.6 Plastic bags (see 8.3.4.2)

5.6 Equipment for the storage of samples

5.6.1 Wide mouth plastic bottles with screw cap

5.6.2 Plastic bags

Eventually with seals (and seal number) if it is to be used in relation with the implementation of EU Regulation 691/2013.

5.6.3 Refrigerator

5.6.4 Freezer

6 Environment and rooms

6.1 Rooms

According to ISO Standard 24276:2006, grinding of samples should be carried out in a room or working area dedicated to that purpose. Special attention should be paid to follow the forward flow in order to avoid contamination.

6.2 Cleaning and decontaminating reagents

All equipment (e.g. mills, grinders, blending devices, sampling tools, balances, laboratory benches, ...) used in the sample preparation procedure must be carefully cleaned upon completing and, if deemed necessary, during sample preparation. Vacuum cleaning alone is not sufficient, a wet cleaning is necessary to eliminate contaminating particles.

Apart from washing solutions such as detergents, it is possible when necessary to eliminate or degrade DNA with the following reagents:

- bleach (e.g. 5-10 v/v % i.e. a 10 to 20 fold dilution of commercial bleach at 12° Chl - in Gay-Lussac chlorometric degrees - or 3.61-3.65 % of active chlorine),
- hydrochloric acid 1N,
- hydrogen peroxide solution at 3 % (US Patent 20070289605 A1, 2007) as well as solutions of several cationic compounds (US Patent US 7687027 B2, 2010),
- ready-to-use DNA removal products (e.g. ELIMINase® of Decon Labs, King of Prussia, Pennsylvania, USA; DNA-ERASE™ of MPBiomedicals, Solon, Ohio, USA).

7 Safety precautions

Appropriate personnel protective equipment (e.g. lab coat) should be worn as required in the laboratory. In an extremely noisy environment (85-90 dB or more), ear protectors (earmuffs or ear plugs) are necessary to prevent ear damage. For noisy equipment, noise reduction enclosures may also be considered.

The mills for crushing, cutting and grinding have sharp blades. One should never put hands or fingers past the edges of the introduction chamber during operation of the equipment nor open the mills until they have completely stopped. It is advised to regularly check that safety interlocks on all equipment are operating properly.

If available, the dust ventilation system has to be operated during dust generation procedures. A vacuum cleaner can for instance be used to minimize dust in the hood area and the working area. One should also be careful when handling (but especially grinding) seeds treated with phyto-pharmaceutical products by wearing appropriate respiratory protective equipment because the dust may be contaminated with these chemicals.

8 Procedure

After registration and check of the laboratory sample (see **8.1**), a first optional step may consist of a representative mass reduction of this laboratory sample (see **8.2**).

In a next step the particles in the test samples are reduced to adequate sizes to minimise the subsampling error arising when the test portion is taken from the test sample (see **8.3**).

For samples with a high moisture content, partial drying prior to grinding could be necessary (see **8.4**).

For liquid samples and some fatty and/or sticky samples (e.g. oil, oilseeds, pet foods, fluid lecithin) special sample preparation procedures are useful / necessary (see **8.5**).

Storage of the samples before and during sample preparation as well as after the analysis should be performed under adequate conditions, be it at room temperature, refrigerated or frozen (see **8.7**).

For particulate material a flow chart of the several steps with a decision tree can be found in **Annex III**.

8.1 Laboratory sample check

Firstly, the laboratory sample will be registered and uniquely identified (e.g. with a unique code number).

Before starting the proper sample preparation procedure some laboratory sample checks have to be carried out.

8.1.1 Check of laboratory sample constitution

Upon arrival in the laboratory the sample should be free from any sign of damage and should be cooled or frozen if prescribed by the shipment conditions. Furthermore, all information concerning the sample should be available and complete.

Deficiencies (e.g. missing important information about the type of sample, an open laboratory sample container, the sampling protocol does not correspond to the sample received) should be documented and eliminated whenever possible.

In case of suspected cross-contaminations between samples, these laboratory samples are not appropriate for analysis. The customer should be informed about the deficiencies in the samples provided.

Table 1 — Recommended laboratory sample sizes according to the type of matrix (Table adapted from AFNOR, 2006).

Products	Recommended laboratory sample size
Seeds	Mass equivalent of 3 000 kernels (see table below for mass equivalent of 1 000 kernels)
Commodity grains	Mass equivalent of 10 000 grains (see table below for mass equivalent of 1 000 kernels)
First transformation products (semolina, flour, grits, oilcake etc.)	From 100 g to 1 kg
Liquids	500 ml
Doughy and viscous products	500 g
End products (e.g. packed rice noodles)	From 100 g to 1 kg

NOTE: The numbers provided are indicative. In special cases other quantities might be required. Some legislative texts specify other values for the laboratory sample size (e.g. for rice as a commodity at least 960g are required according to Commission Decision 287/2013).

Another point to consider during laboratory sample check at its reception is the sample size. **Table 1** provides a list of recommended laboratory sample sizes.

The received laboratory sample size should be stated in the analysis report. It should be stated when the sample size is below the recommended minimal sample intake.

Just as an indication, **Table 2** provides information on what is the average mass of 1 000 kernels of cropped plants that might have an interest with relation to GMO detection.

Table 2 — Data concerning the mean mass of 1 000 kernels for different plant species — data derived from AFNOR (2006), Belitz et al. (2009), the Canadian Grain Commission (2013) and Hübner et al. (2001).

Plant species	Mean mass of 1 000 kernels (in g)
Barley	37
Linseed	6
Millet	23
Oat	32
Maize	285
Rapeseed	4
Rice	27
Rye	30
Soybean	200
Sugar beet	11
Sunflower	100
Tomato	4
Wheat	37

NOTE: be careful that for some species, especially maize, these average values are just an indication and it is better to really establish the mass of a sufficient number of kernels to determine the mass equivalence of 1 000 kernels for that specific variety than to rely on these average values.

Annex IV provides a very useful way to determine what minimal sample size to be taken into consideration when it consists of kernels and that one wants to check that presence of a GMO is below a certain level with a given probability. The outcome should not necessarily be analysed at once, you might do it in subsamples as long as you know in which amount of seeds one transgenic seed can still be detected with your complete method (going up to the PCR results).

There are of course limitations to the use of that statistical approach. It is not usable for potatoes, neither for some fresh fruits (e.g. papaya). This is however briefly considered in the conclusion.

8.1.2 Maximum mass of the laboratory sample

Apart from minimal size requirements of the laboratory sample, it is also advisable to fix a maximum size. This will enable avoiding the mass reduction step on a large quantity of laboratory samples. Maximum numbers should not be fixed in a single unit for all samples but should be adapted to the kind of matrix analysed.

8.2 Mass reduction

When a laboratory sample needs to be ground (for examples of matrices that don't need to be ground see **Annex V**), it is recommended to use the whole laboratory sample in as far as possible. The mass reduction process used has to be documented when only a part of the laboratory sample is used. Several subsampling procedures for mass reduction are outlined below (see **8.2.2**). However, in some cases a pre-grinding step has to be carried out before any mass reduction (see **8.2.1**).

8.2.1 Coarse grinding (or pre-grinding)

When a 'dry laboratory sample' is composed of lumps or its particle sizes are above 6 mm the whole laboratory sample should be pre-ground (e.g. using a cutting mill, or chopped to particle sizes in the range of 4 to 6 mm) before mass reduction / subsampling.

8.2.2 Subsampling procedures

From a mere statistical viewpoint, mass reduction with the use of dedicated devices such as rotational dividers or riffle splitters is generally recommended because the resulting sampling errors are reduced (Petersen et al., 2004). The problem with most of these devices in the case of GMO detection is the

risk of cross-contamination. That is why the approaches which are generally considered to be more simplistic and less efficient are those most frequently applied. All these techniques include incrementing.

Unfortunately, it is common practice to take only a limited number of increments. When a small number of increments is selected, there can be very large subsampling errors due to sample heterogeneity. The number of increments has to be determined in view of the size of the error deemed acceptable and not according to the ease of handling. As a rule of thumb it is recommended to take 10 increments when there is no prior knowledge about the sample heterogeneity.

8.2.2.1 Fractional (alternate) shovelling

It is a very simple splitting technique with the following advantages:

- No costly equipment involved (e.g. riffle splitters);
- Minimal clean up and decontamination requirements;
- Can be implemented in the lab or field;
- Any number of splits can be generated;
- Very low sample splitting error.

The laboratory sample is split into the desired number of samples by collecting increments from the laboratory sample. Increments that should be of identical sizes are alternately placed in containers or piles to create split subsamples.

When a sample is to be split into two equal subsamples, one split would contain the odd increments and the other subsample would contain the even increments (= alternate shovelling). Fractional shovelling implies at least three splits.

When a sample is to be split into three subsamples, the first split would contain increments 1, 4, 7, the second split would contain increments 2, 5, 8, and the third split would contain increments 3, 6, 9, ... (see **Figure 6**)

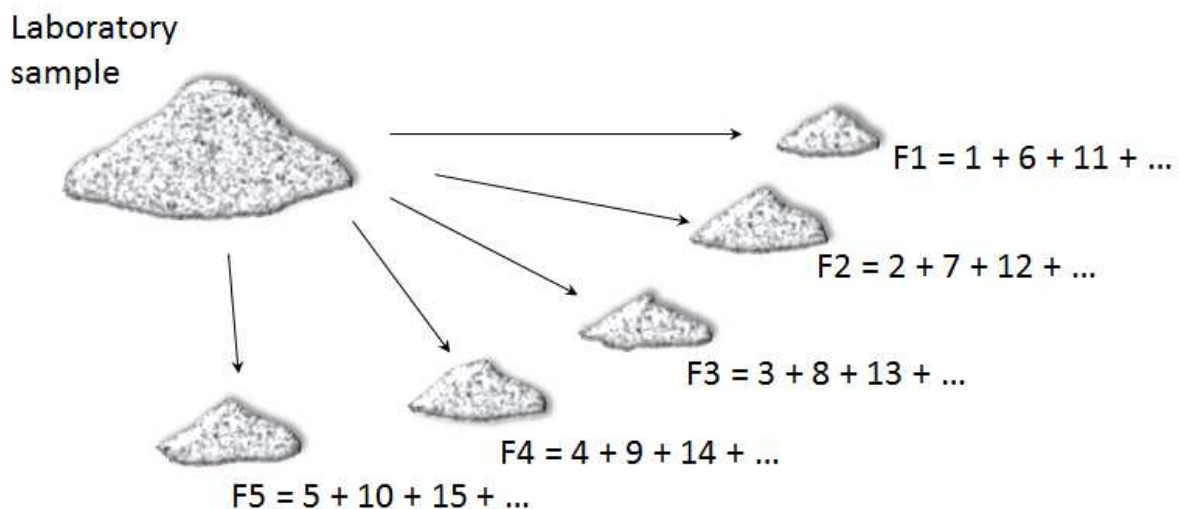


Figure 6 — Illustration of fractional shovelling from a laboratory sample into five subsamples (F1 to F5). (Figure adapted from Gerlach et al., 2002).

The same pattern as the one described above has to be followed when a large number of split subsamples are considered.

The following formula can be used to calculate the mass increment:

$$m_{(\text{increment})} = m_{(\text{lab sample})} / [n_{(\text{subsamples})} \times N_{(\text{increments})}] \quad (1)$$

where m = mass and n and N = number

The following precautions must be considered:

- All increments must have approximately the same size;
- Each subsample must have an identical number of increments;
- Split subsamples must be randomly selected;
- All material must be used.

NOTE: Towards the end of the splitting process there can be a small amount of very small particles called fines; it is advisable to reduce the increment size so that the fines are equally apportioned among the splits; when this is not done, it is possible that all fines will end up in only one of the subsamples.

8.2.2.2 The spoon method (based on Petersen et al., 2004)

The spoon method can be used when a relatively low sampling ratio is needed (reduction by less than a factor 10). The method is somewhat related to bed blending. The laboratory sample is spread out in an even layer. To do so the laboratory sample has to be poured out as a thin layer (< 2 cm) in an S-shape movement in one direction (**Figure 7A**). This is subsequently repeated in the traverse direction (i.e. perpendicular to the first one — see **Figure 7A**). If needed the procedure is repeated until the laboratory sample is poured into the tray but the thickness of the entire layer or bed should reach at least 3 to 4 cm. After the spreading step, the spoon method simply consists in extracting randomly spread increments (**Figure 7B**) using a spatula and a square-edged shovel ensuring that for each of these increments the bottom of the tray is reached. The combination of the several increments will lead to a subsample. The method can be greatly user-dependent but with the above-mentioned guidelines the user-dependency should be reduced.

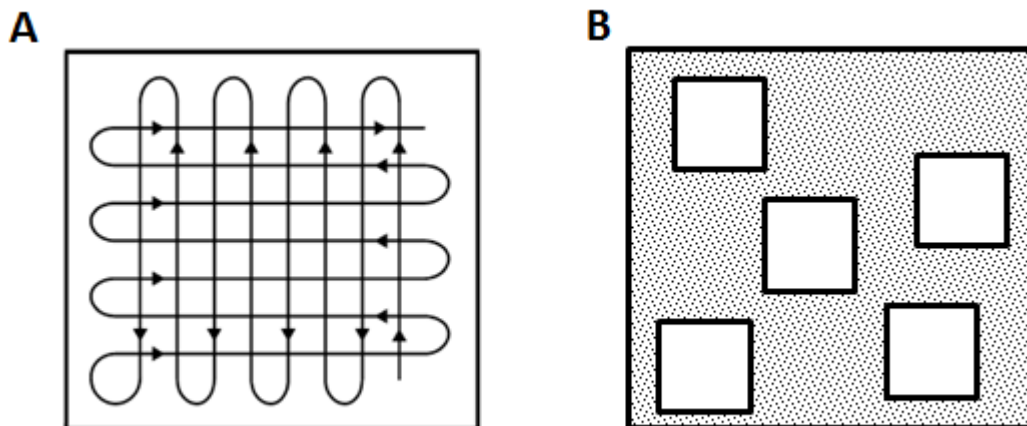


Figure 7 — Mass reduction with the spoon method. A) The ‘S-shaped’ pattern used for spreading the laboratory sample in a tray when performing mass reduction by the spoon method (adapted from Petersen et al., 2004). B) Random spread location of the extracted increments.

8.2.2.3 The long pile method ⁽¹⁾

In this method the sample is poured out of the bag and spread on flat surface in a long pile (**Figure 8A**). The pile should have a length of at least 20 times its width and the height should not exceed the width of the pile. Several cross sections are then taken out of the pile (**Figure 8B-C**) and all the cross sections put together (**Figure 8D**) lead to a mass reduction of the original sample.

The best is to take a sufficient number of not too large sections. The several cross sections are not distributed with the same distance in between each of them (as ideally this should be at random). If

⁽¹⁾ Based on a discussion with K. Esbensen during a training course on sampling given at CRA-W (Gembloux, Belgium) in March 2012.

nothing is known about heterogeneity it is advised to take at least ten cross sections. Like the spoon method, this technique is advised when a low sampling ratio (less than a factor 10) is needed. Basically the technique tries to mimic on a static sample what happens with a sample in motion where cross sections are taken at a given place. Here the sample is static and the cross sections are dispersed along the pile.

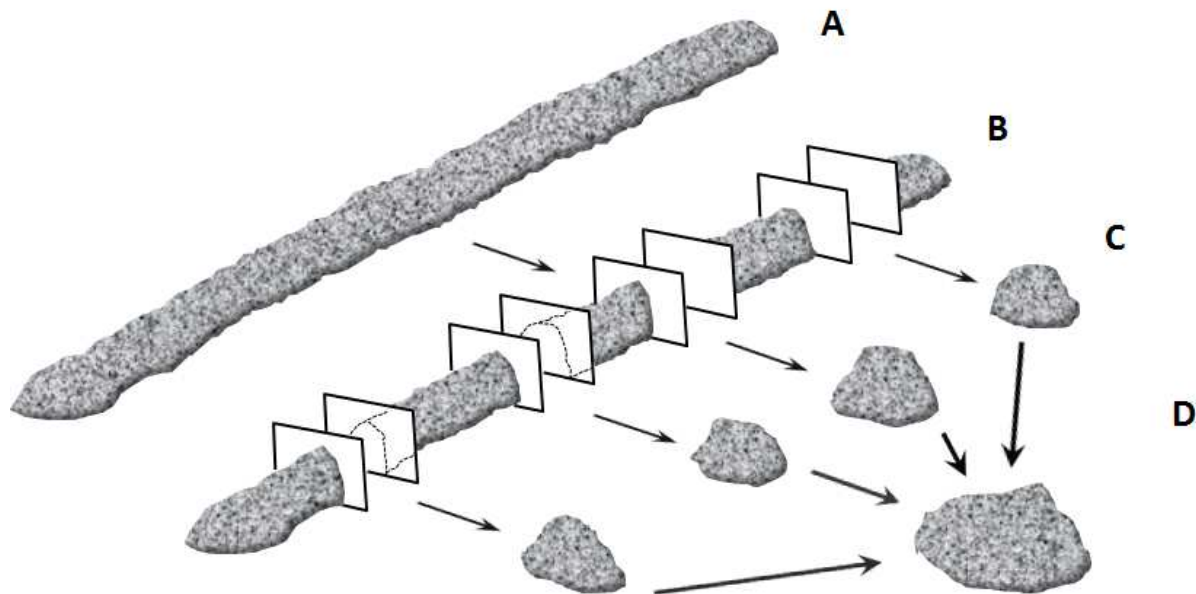


Figure 8 — Schematic representation of mass reduction by the long pile method.

8.2.2.4 Other mass reduction techniques

A separate section is added for other mass reduction techniques that are not favoured by the working group as they might have some disadvantages. However if they are used while correctly understanding the limits of the technique and the results are still satisfying there is no reason not to use them.

The techniques are briefly outlined here:

Coning and quartering: is an old technique that according to literature (but never on matrices for GMO analysis) has a poor performance (Mullins and Hutchison, 1982; Gerlach et al., 2002). Basically it does comply with the fundamental rules required to avoid incorrect sampling errors. So other than the warning that it might have a low performance (i.e. it would deliver subsamples on which there is a too large variation; essentially because incrementing is low) which means in other words that parameters measured on such subsamples would have a greater measurement uncertainty, the technique has the big advantage to be very simple and cleaning is easy to carry out. More detailed explanation on coning and quartering can for instance be found in the ISTA guidelines (2013).

Techniques with more elaborate devices: there is wealth of much more elaborate devices, some being automatic (revolving splitters, rotational dividers, ...) and some not (riffle splitters).

The very big disadvantage of such devices is the risk of contamination as once used it is difficult to get rid of the material that was put in it. Cleaning is therefore generally rather difficult to carry out or is time-consuming and therefore not cost-efficient. There is one exception that should however be mentioned among these devices, which is the riffle splitters. No one in the working group had experience with such devices. It was only when the working group was ending that at least one of the labs had to consider such a device to implement regulation 691/2013. More detailed information on splitters can be found in the ISTA guidelines (2013) as well as in Petersen et al. (2004). **Annex VI** gives an example of what could be a paper riffle cone splitter which might be a suitable device but that does not exist commercially.

8.3 Particle size reduction

Some samples might not require any grinding (for a list of examples, see **Annex V**).

8.3.1 General methods

Comminution, the generic term for particle reduction can be achieved through several processes (list based on ISO 6498:2011):

- Chopping: a material is mechanically cut into smaller parts;
- Crushing: applying pressure to particles to fragment them into smaller particles; especially for samples with large and hard lumps,
- Cutting: cutting mills reduce soft to medium-hard and fibrous materials using rotating and stationary cutting knives;
- Blending (homogenising): materials that are generally semi-solid (i.e. with a certain water content) are mixed with rotating knives which break fragments in smaller particles and blend the entire sample to a macroscopically more uniform texture (at least in appearance) and consistency (e.g. 'Waring blenders');
- milling / grinding: reduces solid particles by a combination of cutting, shearing and pressure;
- Pulverising: describes the action of various mills which further reduce small-sized material (< 10 mm) to an average particle size usually below 75 µm.

8.3.2 Requirements regarding the choice of size reduction equipment

The requirements for suitable size reduction methods differ widely and depend on the sample material. To keep track of possible cross-contamination between successive samples processed by grinding equipment, a logbook should mention the order in which samples have been handled on a specific device.

Considerations for selecting a size reduction equipment for a specific application include the following aspects (list based on ISO 6498:2011):

- What are the physical (e.g. hardness) and chemical properties (e.g. composition, very fatty or not) of the material? Is the size reduction process affected by heat generation, moisture change or chemical reactions?;
- Maximum allowed initial particle size (e.g. chunks, powder, etc.);
- Final desired particle size range (mm, µm);
- Quantity of material to be ground and number of laboratory samples to be processed daily or weekly (taking into account also the time for cleaning the grinder);
- Importance of sample loss due to adherence to the grinding surface;
- It is essential that all parts of the device in contact with the sample are washed completely using decontamination solutions (only vacuum cleaning will not be sufficient);
- The choice of a grinding device should also take account of the possibility to periodically check the grinding efficiency (see examples in **Annex II**).

Other general considerations for grinding are:

- Devices with integrated sieves will give some control of the particle size independently of the kind of matrix processed;
- When successive grinding steps have to be applied with sieves of decreasing mesh sizes, a factor 4 between successive mesh sizes should be used as a rule of thumb: e.g. a first grinding with a sieve at mesh size of 2 mm followed by a subsequent grinding with a sieve at the mesh size of 0.5 mm;
- Especially for fatty samples, it may be difficult to make use of sieves of a mesh size of 0.5 mm as this might generate a heating of the sample which could be detrimental for the DNA content. In such cases, it is advisable to avoid grinding at a mesh size of 0.5 mm but to replace it by a second grinding at a mesh size of 2 mm. As illustrated in **Table 3**, on two kind of rice products, repeated grinding with the same mesh size sieve may lead to results comparable to a first grinding at the mesh size of 2 mm followed by a second one at the mesh size of 0.5 mm;

- Mind that particle size may have an influence on DNA yield during extraction (e.g. Moreano et al., 2005);
- Wet milling is not appropriate for GMO analysis. According to Corbisier et al. (2005) DNA degradation occurred after exposure of ground seed powder to water. DNA degradation is proportional to the vigour of the mixing process. It can be assumed that in the slurry the DNA is enzymatically degraded by endonucleases released by the cells.

NOTE: There is no industry standard for classifying particle size reduction equipment. A comprehensive summary of several types of grinders is provided in ISO 6498:2012.

Table 3 — Percentage of recovery of ground rice products according to the granulometry. Grinding was performed on a ultracentrifugal mill with sieves at a given mesh size. A single passage or several passages through the 2 mm mesh size sieve is compared to a passage with the 2 mm mesh size sieve followed by another passage through the 0.5 mm mesh size sieve (data from Janssen, 2013).

Sample	Grinding (Retsch ZM200)	Percentage of recovery according to the granulometry			
		> 0.5 mm	[0.5 — 0.25] mm	< 0.25 mm	≤ 0.5 mm
Rice kernels 1	1 x 2 mm sieve	17.5	38.6	43.8	82.4
	2 x 2 mm sieve	3.4	30.0	66.4	96.3
	3 x 2 mm sieve	0.6	17.3	81.9	99.2
	1 x 2 mm sieve + 1 x 0.5 mm sieve	0.2	10.1	89.7	99.7
Rice kernels 2	1 x 2 mm sieve	19.6	40.8	39.5	80.3
	2 x 2 mm sieve	4.2	33.7	62.2	95.8
	3 x 2 mm sieve	0.8	19.5	79.9	99.3
	1 x 2 mm sieve + 1 x 0.5 mm sieve	0.3	11.5	88.2	99.7
Rice noodles 1	1 x 2 mm sieve	20.4	43.4	36.4	79.8
	2 x 2 mm sieve	3.1	33.0	64.0	96.9
	3 x 2 mm sieve	1.3	26.7	72.0	98.7
	1 x 2 mm sieve + 1 x 0.5 mm sieve	0.1	16.4	83.4	99.8
Rice noodles 2	1 x 2 mm sieve	21.2	43.1	35.7	78.8
	2 x 2 mm sieve	4.8	37.3	58.0	95.3
	3 x 2 mm sieve	1.3	28.0	70.8	98.8
	1 x 2 mm sieve + 1 x 0.5 mm sieve	0.3	21.3	78.6	99.9

8.3.3 Maintaining integrity of the laboratory sample

To minimise the loss of integrity of DNA molecules of the sample, heating during the grinding process must be limited (e.g. when grinding some fatty matrices like linseed with a 0.5 mm mesh size sieve).

8.3.4 Mixing techniques

8.3.4.1 General considerations on mixing techniques

Mixing is used by many laboratories in an attempt to 'homogenise' the sample. Once the sample is 'homogenised', any increment or grab from the sample is then deemed representative of the sample without any further considerations. This assumption does however not hold true for particulate matter.

Annex V gives a list of matrices that are considered as homogenous and therefore don't need any mixing technique before taking the test portion.

While mixing may be possible in some cases, it might actually promote segregation in other cases. This is especially true for material with diverging particle sizes and densities.

One type of mixing technique which is strongly discouraged is putting a spatula in the top of a sample container and stirring it a few times vertically.

A second unacceptable technique for dry, ground material is shaking a sample container when the jar is full or almost completely filled. The material at the bottom of the container is not properly mixed in and the stirring might have actually promoted segregation thus enlarging the mass reduction error. It is recommended to leave at least one third of open space in the jar.

There are many other mixing techniques available which are superior to stirring (e.g. drum mixer, stirring vane mixer, V-shaped double cylinder mixer, overhead mixer, tumbler mixer). One practical technique carried out in a laboratory of one of the Working Group members is outlined hereafter.

8.3.4.2 The plastic bag technique

The ground material is put in a plastic bag that can be closed (sealed, zipper). Leave approximately one half of open space in the bag. Homogenization occurs through 20 successive reversals of the bag (**Figure 9**).

Although it may depend on the nature of the material, for commonly treated samples the following bag sizes can be considered:

- for 1 kg of ground sample: a plastic bag of 30 cm by 40 cm,
- for 2 kg of ground sample: a plastic bag of 37 cm by 50 cm.

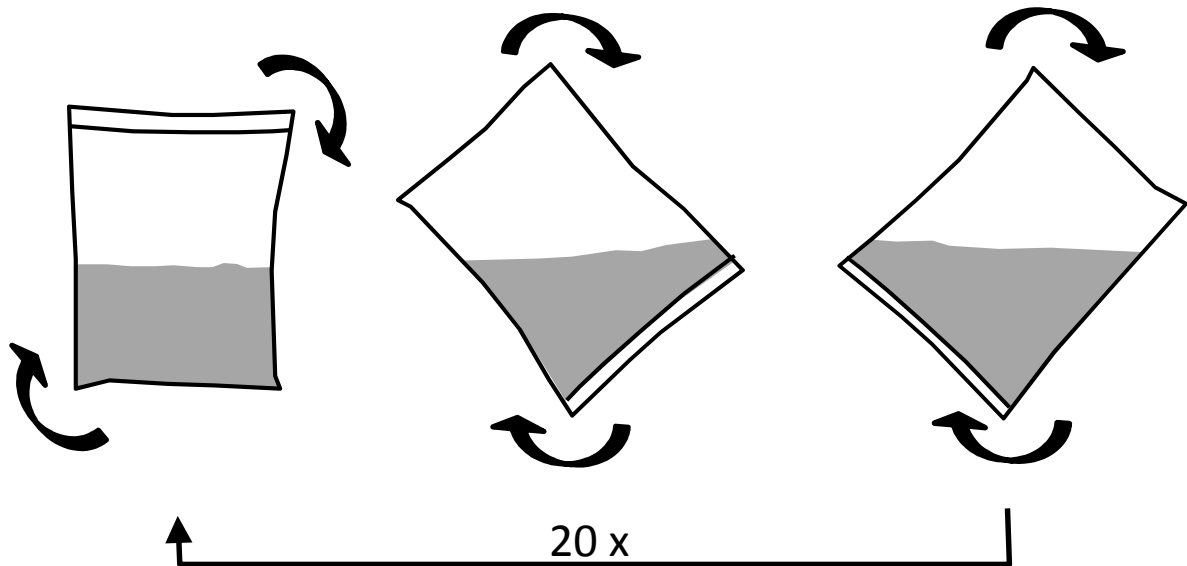


Figure 9 — Homogenisation by mixing with the plastic bag technique.

8.4 Partial drying

For some semi-solid matrices with less than 85 % dry matter (e.g. forages, fresh bread, but not for liquids), it is necessary to partially dry them prior to fine grinding.

Partial drying can be done using either a forced-air oven or a microwave oven. The aim is to dry the sample while keeping its temperature below 55–60 °C to minimally affect its chemical composition. Drying at higher temperatures (i.e. above 60 °C) might cause chemical changes which could affect the DNA content of the sample. Following drying, the subsample is ground.

8.5 Special sample preparation procedures

8.5.1. Gelatine, molasses and samples with high fat content

For samples composed of high contents of fat, gelatine, and / or molasses, some special sample preparation procedures help in obtaining a representative subsample and finely ground test samples:

- Cool the entire laboratory sample in a freezer overnight and process the sample in a frozen or chilled state;
- Use dry ice during grinding to keep the sample cold enough to prevent clumping or melting;
- Use blending type mills, operated in intervals of 30 s.

8.5.2 Viscous samples

For viscous samples such as fluid lecithin and honey heating the sample at 40–60 °C improves the homogeneity of the sample and helps obtaining a representative sub sample.

8.5.3 Liquid samples

Liquid samples are generally considered as homogeneous (Ministero della Salute, 2012) and do not require prior homogenisation. However, there may be exceptions like for oil where DNA is present in solid particles that may be in suspension in the liquid. In such a case it seems preferable to use a method described by Costa et al. (2010) where the laboratory sample is submitted to a centrifugation of 30 minutes at ~ 18 000 g. The supernatant is discarded and DNA extraction is performed on the pellet.

8.5.4 Plant tissues

For plant tissues, two possibilities can be considered:

- A technique which generally works well on fresh tissues is to freeze the material in the presence of liquid nitrogen using a mortar and pestle or eventual other disruption devices to crush the material;
- An alternative to the liquid nitrogen technique consists of freeze-drying the material (which might take more than one day) and subsequently grinding the dried material.

8.6 Test portion

8.6.1 Homogenisation of the test sample (or analytical sample) prior to test portion uptake

Most solid samples will first be ground before taking the test portion from the homogenised test sample or analytical sample. It is however possible to store the homogenised material for a while before the test portion uptake. When proceeding in such a way to include a resting period for the homogenised test sample, it is advisable to homogenise this sample by agitation of the jar in which it was kept (hence requiring at least one third of available volume) before the uptake of the test portion. Special equipment (e.g. overhead mixer) can be used for this purpose.

8.6.2 Minimum mass of test portion

To properly represent a laboratory sample, the test portion (i.e. the final subsample submitted to the further analytical process) must contain an adequate mass in accordance with the fundamental subsampling error (FSE) and maximum particle size ('minimum mass'). The test portion mass should increase by a factor of 8 when the particle size doubles to maintain the same representativeness of the laboratory sample (**Table 4**). This is because the test portion size is proportional to the power 3 of the particle size. It clearly shows that if the maximum particle size exceeds 3 mm, this will lead to an excessive fundamental sampling error when taking into account that the maximum test portion size for DNA extraction is of 5 g (Terry et al., 2002). As most generally the test portion size does not even exceed 2 g it is advised to be below 2 mm as a maximum size of particles.

Because of technical limitations and reagent costs, the sample intake for DNA extraction is relatively small. This implies that special care must be devoted to obtain a sufficiently small size of particles to be integrated in the test portion; otherwise errors linked to constitutional heterogeneity of the material will become too high.

Other tables (**Table 5 A** and **B**) concerning the test portion size are based on data from Berben and Janssen (2008) that are derived from Lischer (2001b). They indicate the minimal test portion required to detect a certain threshold amount of GMO in a given context of sampling error. It should be mentioned that the figures are theoretical. Moreover in practice it is difficult to estimate the size as well as the weight of a particle (Janssen et al., 2010).

Table 4 — Theoretical calculations of the minimum mass of the test portion: expected relative standard deviation (RSD) linked to the maximum particle size (assumed density of 1 g/cm³) — data adapted from ISO/FDIS 6498:2011. The yellow cells are those compatible with common sample intakes for DNA extraction (maximum 5 g).

FSE (expected RSD)→	25 %	20 %	10 %	5 %	2 %	1 %
Maximum particle size ↓						
0.5 mm	0.02 g	0.03 g	0.13 g	0.5 g	3 g	12.5 g
0.75 mm	0.07 g	0.1 g	0.42 g	1.7 g	10.5 g	42 g
1 mm	0.16 g	0.25 g	1 g	4 g	25 g	100 g
2 mm	1.28 g	2 g	8 g	32 g	200 g	800 g
3 mm	4.3 g	6.7 g	27 g	108 g	672 g	2688 g
4 mm	10.2 g	16 g	64 g	256 g	1600 g	6400 g

NOTE: for materials with densities other than 1 g/cm³, this table can be modified by multiplying the minimum mass with a corrective factor corresponding to the density of the material expressed in g/cm³.

Table 5 — Minimum test portion in mg to reach a sampling reproducibility (at a 95 % probability level) of 20 % **A**) if grouping and segregation error and also analytical error are assumed as negligible, **B**) if grouping and segregation error is considered equivalent to the fundamental error and if the analytical error is set at 5 % (from Berben and Janssen, 2008 based on data from Lischer, 2001b).

A	Critical GMO content			
	0.1 %	0.5 %	0.9 %	1.0 %
Mean mass of particles				
5 µg	500 mg	100 mg	55 mg	50 mg
10 µg	999 mg	199 mg	110 mg	99 mg
20 µg	1 998 mg	398 mg	220 mg	198 mg

B	Critical GMO content			
	0.1 %	0.5 %	0.9 %	1.0 %
Mean mass of particles				
5 µg	499.5 mg	265 mg	147 mg	132 mg
10 µg	2 664 mg	531 mg	293 mg	264 mg
20 µg	5 328 mg	1061 mg	586 mg	528 mg

Table 6 gathers experimental data collected during the Co-Extra project (Janssen et al., 2010). Samples consisting of soybean with known amounts of genetically modified soybean kernels were tested at different test portion sizes. The DNA extraction method used was checked to provide enough DNA within a PCR even with the smallest test portion to make detection at the lowest level possible but due to random distribution of ground material (constitutional heterogeneity) the outcome is that the relative standard deviation on the measured content at 0.1 % for most of the test portion sizes considered is above 25 %. This result has a consequence for quantification of low level presence event quantification. The test portion size considered on the tested sample should be large enough because otherwise the measurement uncertainty is unacceptable with respect to EU Regulation No 619/2011.

Table 6 — Experimental data collected during the Co-Extra project on test portion size for detection of genetically modified soybean in soybean kernels (Janssen et al., 2010). An exclamation point indicates for what combination of test portion size and GMO content the relative standard deviation on the measured content exceeded 25 %.

Test portion size	GMO %						
	0.1 %	0.3 %	0.6 %	0.9 %	1.2 %	1.5 %	1.8 %
50 mg	!	✓	✓	✓	✓	✓	✓
100 mg	!	✓	✓	✓	✓	✓	✓
200 mg	!	✓	✓	✓	✓	✓	✓
400 mg	!	✓	✓	✓	✓	✓	✓
800 mg	✓	✓	✓	✓	✓	✓	✓

8.6.3 Test portion uptake

The test portion uptake is generally done using so-called grab sampling. Since care has been taken to prepare a rather homogeneous test sample, grab sampling at the level of sample preparation is generally deemed acceptable. As the test portion size is generally determined by weighing, care should be taken to perform this according to good weighing practices. Use of antistatic devices may sometimes be helpful with finely ground material to avoid weighing errors but also to limit contamination of the balance.

8.7 Storage and disposal

Once a representative test sample has been prepared from the laboratory sample, it is essential to maintain its integrity throughout the analytical and post-analytical phases (i.e. including all analytical processes and reporting of data) until the ultimate disposal of any left-over material.

A proper storage may include cooling (i.e. refrigeration or freezing), controlled humidity conditions to protect samples from moisture gain or loss, protection from UV light, etc. The aim of these storage conditions is to prevent the breakdown of organic compounds by an excess of harmful microorganisms.

The proper storage conditions will depend on the various types of matrices to be analysed. One should bear in mind that the storage of the laboratory sample and of the reserve sample can be completely different. For example fresh papayas can be kept some days at room temperature or in a refrigerator whereas the reserve sample which has to be kept for some months will have to be frozen.

Storage and disposal policy should be established and documented by the laboratory.

When the matrix consists of living GMOs (grains, kernels, seeds) care should be taken to avoid their dissemination into the environment upon elimination of this material (e.g. the whole sample should be ground).

9 Performance tests (quality control)

Performance tests are described to estimate the sample preparation error, the latter depending on the procedure, the material, the analytes and the analyst.

Performance tests can be used to evaluate new equipment by comparing results obtained with the new and the previous equipment.

Some performance test procedures are given below. These procedures have to be adapted to meet the laboratory needs.

9.1 Performance test for particle size reduction (grinding)

9.1.1 Grinding quality

When using centrifugal grinders with sieves at defined mesh sizes several regular performance tests (e.g. on an annual basis) can be designed:

- It is generally considered that for a given mesh size of a sieve in a centrifugal grinder, the ground product should consist of at least 80 % of particles below half of the mesh size;
- Due to repetitive use, the mesh size of a sieve in a centrifugal mill increases with time. Its proper functioning is confirmed when at least 95 % of the material ground is below the nominal mesh size of the sieve.

For performing these tests the ground material has to be analysed using calibrated sieves. Not every matrix is suitable for that purpose. Ground soybeans give rise to a fatty and sticky material which is very difficult to analyse with calibrated sieves. A good matrix for these performance tests is e.g. wheat or rice. It is recommended to store a wheat lot intended for performance tests in order to have comparable figures over several years. It will for instance become evident that the proportion of particles exceeding the nominal mesh size of the sieve will progressively increase due to the repetitive use of the sieve.

9.1.2 Carry-over

9.1.2.1. Negative sample test

A way to check the prerequisites of the cleaning process of a mill and its correct execution by different operators consists in analysing a negative sample following a positive sample with preferably a rather high content of genetically modified material (> 5 %). Alternatively the test can be done on different species (e.g. soybean and maize). The cleaning process will of course have been carried out in between samples.

The major difficulty with this technique however for genetically modified material is that one can never be completely sure of the real negative character of the so-called negative sample (for more details linked to how to check this, see **Annex IV**). The laboratory has to decide about the frequency of the test and the acceptance criteria.

9.1.2.2. IKI carryover test

This is another example of a carryover test that is fully described in ISO 6498:2012 and which is probably of less interest with respect to the scope of this guideline. It consists to check if starch particles could contaminate another sample by colouring the starch with a lugol solution (IKI for iodine-potassium iodide solution). The ISO 6498:2012 standard accepts 0.25 % (m/m) of contamination which for GMO analysis is not acceptable.

9.2 Performance test for mixing

Grinding is often a source of segregation, because particles which are more difficult to grind may not pass the sieve as quickly as the softer particles. Improper mixing will only emphasise this problem.

The mixing container should never be more than two-thirds full and preferably only half full. An example of a performance test for mixing is given hereafter.

Unmixed layers method

One fourth of the container volume of the normal storage container is filled with ground wheat or a similar light-coloured material. One fourth volume of a ground coloured mineral mix is added on top (taking into consideration realistic densities of test material which are routinely analysed). The content of the container is mixed. The time needed to mix the contents until they are visually blended is recorded. This unmixed layers method can be used to test any mixing method in the lab.

9.3 Environmental control

ISO 24276:2006 considers the possibility of using environmental controls.

The other controls prescribed for the PCR analysis by ISO 24276 don't cover the sample preparation step. The environmental control is a possible mean to regularly check the cleanliness of the air in the grinding room or any other workspace linked to sample preparation considered to be critical for the environmental control described in ISO 24276:2006.

9.4 Representativeness of the test portion

As the test portion used in GMO analysis is always small, it is essential to check that its size combined with the grinding technique used ensures a sufficient representativity of the sample within the test portion. A way to do this is to check on kernels (GM — non-GM or different species) if a level somewhat above the limit of detection can be reliably detected during replicate analyses on the ground material: e.g. analyse six test portions and all should deliver a positive result. Similarly for quantitative analysis when a level somewhat above the limit of quantification is analysed, the results of a replicate analysis on the test portion should deliver a relative standard deviation that does not exceed 25 %. If the performance level is not reached it means that either the test portion should be increased or that the mean particle size of the yielded ground material should be smaller.

9.5 Overall performance test on sample preparation steps through a replication experiment

A way to evaluate the variability of the entire sample preparation procedure can be done through a replication experiment as suggested for sampling strategies by Esbensen and Julius (2010). The interest of this approach is that it also points out per step from where the largest variability is coming. An example is outlined in **Annex VII**.

10 Identified gaps and conclusions

The present document set up a global framework for the several actions involved in sample preparation during GMO analyses. To that aim it could benefit from an already existing structure laid down in a standard intended for feed samples. It was adapted to a specific use for GMO analysis but with an enlargement of the matrices considered as next to feed, seeds, food and environmental samples were also to be taken on board. The existing text is useful for most of the common samples a laboratory could get but it is impossible to be complete and consider all cases that might need a special handling. That is also why a reminder of the fundamental principles of sampling and the errors associated with it are crucial as it allows considering if for a given special matrix the procedure intended to be followed will not generate incorrect sampling errors.

This document that is the deliverable of the working group is meeting the general expectations of the mandate set by the ENGL steering committee. However, it still is more a catalogue of possibilities that can be considered with some highlights on possible caveats or errors that should be avoided than a real list of fully documented standard operation procedures that are directly usable by a technician. Intended readerships are more the laboratory responsables than the technicians. In that sense the deliverable was unable to meet some of the more specific aims of the mandate but this is due to the fact that the so set aim was simply too ambitious. Another example is giving some weight to several steps in the final measurement uncertainty. Doing this was simply impossible because it will largely vary between the several types of matrices under analysis. However, a procedure is detailed in **annex VII** on how to perform this if one would like to have an idea about it on a defined matrix.

Because of the fact that it was built on a model of an existing standard, it might eventually be suggested to serve as model for setting up a real standard with that same purpose of sample preparation in the field of GMO analysis. This means that it certainly requires to be regularly updated which then could be a possibility to add progressively more detailed standard operational procedures. Some of the documents it contains might need a larger discussion. For instance, the document of **Annex V** concerning the list of homogenous material or of material that does not require grinding is coming from the Italian authorities. It might require some enlargement or even revision of some of the existing items that it is listing.

One of the essential aims of the document especially through some of the data provided is to attract the attention of the reader on the fact that sample preparation is probably the step that at laboratory level will account for most of the measurement uncertainty on the result that is delivered. An easy example for that is to be found in Table 4 on test portion size in relation to main particle size of the sample. It clearly shows that if the mean size of the particle is not below 4 mm (and we know some laboratories don't grind with sieves below 4mm), this would require to perform the analysis on a test portion with a much larger size than what is commonly considered, just to keep the uncertainty within an acceptable range. So if this text would just open the eyes of some persons in the sense that some of the procedures they use generate too large measurement uncertainties, it would already be a large success.

Among the gaps that could be identified in setting up these guidelines is the fact that at several occasions ten replicates have to be considered but without a real scientific underpinning of this figure. For sure there is a difference between already homogenous samples and more heterogeneous ones and the figure of ten is probably a kind of compromise. Nevertheless it is not even sure that on really widely heterogeneous samples this figure of ten is really sufficient. The only way to check this would be to perform more practical research on this kind of topic but this was not part of the mandate. We just kept our action to available data.

Another difficulty that was met is that statistical considerations sometimes arrive to figures that are scientifically right but nevertheless unrealistic for practical application. An example for this is that for seeds or kernels either 3 000 or 10 000 kernels are generally advised as an ideal sample size for the laboratory sample. Extending this to potato would of course be an error. Although already challenging, it would certainly be possible to gather the required number of tubers but going on in a cost-efficient way to analyse this laboratory sample remains for time being a quite impossible task. That is why in such a situation one must try to circumvent the problem by alternative approaches. In the very special cases of potatoes which are in some way clones, it seems that an expert easily finds out in the field which plant is different from the others. That is why for potato it is apparently much more practical to work on leaf material collected from the field and focussing on plants that are morphologically different

from others than to work on the commercialized products. Of course such a way of thinking has then to be integrated in control plans of the authorities.

As set in the introduction we considered sample preparation here as all operations between the laboratory sample and the test portion. It should be stressed that subsampling as such may still exist afterwards (e.g. during the DNA extraction step if for instance only part of the material is considered to go through the process). Moreover, most of the principles outlined in the guideline remain valid before reaching the laboratory sample. Therefore the paragraphs on mass reduction, on homogenisation or coarse grinding of this guideline may also be of interest to those that have to make final samples from aggregate samples as requested by EU Regulation 691/2013 that entered into force for feed analysis since the 1 January 2014. Nevertheless it was not the aim of the working group to focus on that as it is outside the mandate. As a consequence someone wanting to use this guideline for that purpose may not find an answer to all his questions.

Finally we also hope this document may help to better harmonize the practice in sample preparation as a survey carried out during the Co-Extra project showed that there was a very large variety of actions among labs (Berben et al., 2010).

11 Bibliographic references

AFNOR (2006), *Implementation guide for standards NF EN ISO 24276, NF EN ISO 21569, NF EN ISO 21570 and NF EN ISO 21571 — Nucleic acid based methods of analysis for the detection of genetically modified organisms and derived products* (in French), Document GA V03-042, 24 pp., AFNOR, France.

Belitz, H.D., Grosch, W., Schieberle, P. (2009), *Food Chemistry*, 4th revised and extended edition. Springer-Verlag, Berlin-Heidelberg, ISBN 978-3-540-69933-0.

Berben, G., Janssen, E. (2008), *Subsampling (or secondary sampling) approaches*, Chapter 10 in CO-EXTRA Deliverable 4.3, 'Existing sampling plans and needs for the development of novel sampling approaches for Genetically Modified Organisms (GMO) evaluation' (Miraglia, M. ed.), pp. 49-60.

Berben, G., Janssen, E., Onori, R. (2010), *Report on the results of the questionnaire on sample preparation*, Co-Extra project, Deliverable D4.6.

Canadian Grain Commission (2013), *Sampling and testing protocol for Canadian flaxseed exported to the European Union*, available at: <https://www.grainscanada.gc.ca/gmflax-lingm/stpf-peevl-eng.htm> (consulted on 20 February 2014).

CEN TS 15568 (2007), *Methods of analysis for the detection of genetically modified organisms and derived products — Sampling strategies*.

Corbisier, P., Trapmann, S., Gancberg, D., Hannes, L., Van Iwaarden, P., Berben, G., Schimmel, H., Emons, H. (2005), 'Quantitative determination of Roundup Ready soybean (Glycine max) extracted from highly processed flour', *Anal. Bioanal. Chem.*, Vol. 383, No 2, pp. 282-290.

Costa, J., Mafra, I., Amaral, J.S., Oliveira, M.B.P.P. (2010), 'Monitoring genetically modified soybean oil extraction and refining processes by polymerase chain reaction techniques', *Food Research International*, Vol. 43, pp. 301-306.

European Commission (2004), 'Commission Recommendation No 2004/787/EC of 4 October 2004 on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC) No 1830/2003', *Official Journal of the European Union*, OJ L 348, pp. 18-26.

European Commission (2009), 'Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed', *Official Journal of the European Union*, OJ L 54, pp. 1-130.

European Commission (2011), 'Regulation (EU) No 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired', *Official Journal of the European Union*, OJ L, Vol. 166, pp. 9-15.

European Commission (2013); 'Commission Implementing Decision 2013/287/EU of 13 June 2013 amending Implementing Decision 2011/884/EU on emergency measures regarding unauthorised genetically modified rice in rice products originating from China', *Official Journal of the European Union*, OJ L 162, pp. 10-14.

European Commission (2013), 'Regulation (EU) No 691/2013 of 19 July 2013 amending Regulation (EC) No 152/2009 as regards methods of sampling and analysis', *Official Journal of the European Union*, OJ L 197, pp. 1-12.

Esbensen, K.H., Julius L.P. (2010), 'Representative sampling, data quality, validation — a necessary trinity in chemometrics', *Comprehensive Chemometrics*, Vol. 4, pp. 1-20.

- Esbensen, K.H., Paasch-Mortensen, P (2010), 'Process sampling: Theory of sampling — the missing link in process analytical technologies (PAT)', *Process Analytical Technology: Spectroscopic tools and implementation strategies for the chemical and pharmaceutical industries* (ed. Bakeev, K.), Second edition, John Wiley and sons, pp. 37-80.
- Gerlach, R. W., Dobb D.E., Raab, G.A., Nocerino J.N. (2002), 'Gy sampling theory in environmental studies. 1. Assessing soil splitting protocols', *J. Chemometrics*, Vol. 16, pp. 321-328.
- Hübner, P., Waiblinger, H.-U., Pietsch, K., Brodmann, P. (2001), 'Validation of PCR methods for quantitation of genetically modified plants in food', *J. AOAC Int.*, Vol. 84, No 6, pp. 1855-1864.
- ISO 24276:2006/Amd 1:2013, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions*.
- ISO 3534-1:2006, *Statistics — Vocabulary and symbols — Part 1: General statistical terms and terms used in probability*.
- ISO 6498 (2011), *Draft version ISO/FDIS 6498:2011(E) of CEN/TC 327 and ISO/FDIS 6498 'Animal feeding stuffs — Guidelines for sample preparation'*.
- ISO 6498:2012, *Animal feeding stuffs — Guidelines for sample preparation*.
- ISO 7002:1986, *Agricultural food products — Layout for a standard method of sampling from a lot*.
- ISTA (2013), *International Rules for Seed Testing*, International Seed Testing Association, Bassersdorf, Switzerland, ISBN 978-3-906549-68-2.
- Janssen, E., Planchon, V., Oger, R., Berben, G. (2010). Assessing the validity of procedures deriving test portion from laboratory sample. In: Deliverable D4.9. Survey, analysis and development of cost efficient sampling plans, Co-Extra project.
- Janssen, E. (2013), *DNA extraction from fresh papayas and rice products. NRL-GMO practical workshop 'sample preparation — DNA extraction from different matrices'*, Brussels, 6 June 2013. Available on: https://intralab.favv-afsca.be/labo/formations/presentations/documents/2-NRL-GMOworkshop_06062013_E.Janssens.pdf (consulted on 18 November 2013).
- Lischer, P. (2001a), 'Sampling procedures to determine the proportion of genetically modified organisms in raw materials. Part I: Correct sampling, good sampling practices', *Mitt. Lebensm. Hyg.*, Vol. 92, pp. 290-304.
- Lischer, P. (2001b), 'Sampling procedures to determine the proportion of genetically modified organisms in raw materials. Part II: Sampling from batches of grain', *Mitt. Lebensm. Hyg.*, Vol. 92, pp. 305-311.
- Ministero della Salute (2012), *Piano nazionale di controllo ufficiale sulla presenza di organismi geneticamente modificati negli alimenti*, 36 pp., available from http://www.salute.gov.it/imgs/C_17_pubblicazioni_1666_allegato.pdf (consulted on 15 May 2013).
- Minkkinen, P.O., Esbensen, K.H. (2009), 'Grab vs. composite sampling of particulate materials with significant spatial heterogeneity — A simulation study of "correct sampling errors"', *Anal. Chim. Acta*, Vol. 653, No 1, pp. 59-70.
- Moreano, F., Busch, U., Engel K.-H. (2005), 'Distortion of genetically modified organism quantification in processed foods: influence of particle size compositions and heat-induced DNA degradation', *J. Agric. Food Chem.*, Vol. 53, pp. 9971-9979.
- Mullins, C.E., Hutchison, B.J. (1982), 'The variability introduced by various subsampling techniques', *J. Soil Sci.*, Vol. 33, No 3, pp. 547-561.

Myers, J. (2004), *Representative subsampling at the laboratory: new guidance from USEPA*, Powerpoint presentation of Washington Group International available on Internet at http://www.dtic.mil/ndia/2004Chemistry/Myers_AFCEE_PresJCM.pdf (consulted on 2 January 2007).

Petersen, L., Dahl, C.K., Esbensen, K.H. (2004), 'Representative mass reduction in sampling — a critical survey of techniques and hardware', *Chemometrics and Intelligent Laboratory Systems*, Vol. 74, pp. 95-114.

Petersen, L., Minkinen, P., Esbensen, K. H. (2005), 'Representative sampling for reliable data analysis: Theory of Sampling', *Chemometrics Intell. Lab. Syst.*, Vol. 77, pp. 261-277.

Terry, C.F., Harris, N., Parkes, N. (2002), 'Detection of genetically modified crops and their derivatives: critical steps in sample preparation and extraction', *J. AOAC Int.*, Vol. 85, No 3, pp. 768-774.

US Patent 20070289605 A1 (2007), *Use of dilute hydrogen peroxide to remove DNA contamination*, (available online: <http://www.google.com/patents/US20070289605>, consulted on 29 October 2013).

US Patent US 7687027 B2 (2010), *leaning compositions, methods and materials for reducing nucleic acid contamination*, (available online: <http://www.google.com/patents/US7687027?hl=fr&cl=en>, consulted on 29 October 2013).

Annex I

Definitions related to the concept of 'Sample' according to standards and European legislation

The comparisons between definitions are summarized in **Table 7**.

Table 7 — Comparison of several definitions.

	Laboratory sample*			Test portion — (Test sample*)
CEN/TS 15568:2007 (E) * ISO 24276:2006	Sample as prepared for sending to the laboratory and intended for inspection or testing [ISO 7002:1986]			Sample, as prepared for testing or analysis, the whole quantity being used for analysis or testing at one time [ISO 3534-1:2006]
Regulation (EU) No 691/2013	A sample intended for the laboratory (as received by the laboratory) and can be the final, reduced or aggregate sample.			
	Laboratory sample	Counter sample	Analytical sample	Test sample
Recommendation 2004/787/EC	Quantity of product taken from the bulk sample intended for laboratory inspection and testing	Sample retained for a specific period of time for enforcement or referee purposes	Homogenised laboratory sample, consisting either of the whole laboratory sample or a representative portion thereof.	According to: CEN/TS 15568:2007 and ISO 24276:2006
	Final sample			Test portion — (Test sample*)
Regulation (EU) No 152/2009 Regulation (EU) No 619/2011 Regulation (EU) No 691/2013	A part of the reduced sample or of the homogenised aggregate sample.			According to CEN/TS 15568:2007 and * ISO 24276:2006

Annex II

Examples of grinders used by laboratories involved in GMO detection

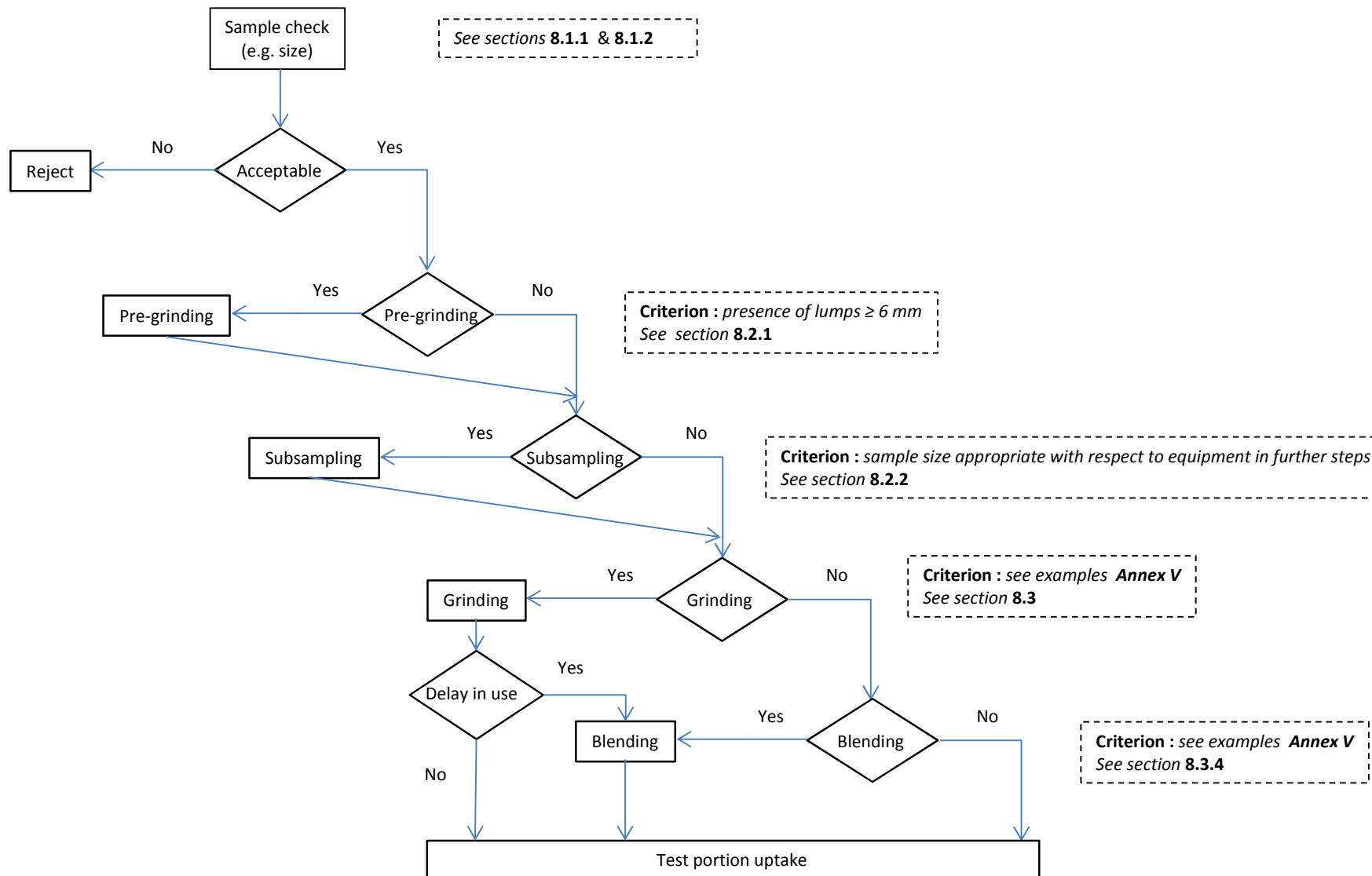
Table 8 — Grinders used in GMO detection (based on a small enquiry among the laboratories participating to the working group — based on 5 replies).

Brand (of the grinder)	Retsch	Retsch	Vorwerk	IKA	IKA	Maxi Grinder	Waring
Type	ZM200	Grindomix GM200	Thermomix 21	A11 basic	M20	Solo	Blender, 1, 2 and 4 l capacities
Maximal capacity that can be ground at once (in grams unless other unit used)	Up to 300 ml with standard cassette (4 500 ml with cyclone)	Up to 700 ml	2 000 (depending on mass and volume)	250 (depending on mass and volume)	500 (depending on mass and volume)	2 000 (depending on mass and volume)	From 200 to 2 000 according to the capacity
Number of laboratories using this equipment	5 (7)*	3	1	1	1	1	1
Is there any integrated system for size control of particles?	Yes (due to sieve)	No	No	No	No	No	No
Easiness of cleaning When a sample is ground. How long does it take to have all cleaned and ready for the next sample?	Difficult 15-60 minutes (depending of the mesh-size, material used); Time reduction when using extra containers/sieves and rotors	Medium 10 minutes Time reduction when using extra blades and bowls	Simple 1-5 minutes (pot, lid, blade) Time reduction when using extra pots/blades and dishwasher	Simple 3-5 minutes	Medium 5-10 minutes	Simple 3-5 minutes Time reduction when using extra containers/blades and dishwasher	Medium 10 minutes
Are there matrices that do not fit?	Oily and fatty material (e.g. linseed), depending on the mesh-size (difficult with sieves below 1 mm); Particles of sample should not exceed 10 mm.); Not suitable for very hard material as well as pasty material	Not suitable for very hard material	Soft/elastic material (e.g. fresh leaves)	Soft/elastic material (e.g. fresh leaves)	Soft/elastic material (e.g. fresh leaves)	Soft/elastic material (e.g. fresh leaves)	Matrices with high content of fat are difficult to grind; the grinding speed must be decreased
Additional remarks (if any)	Additional mixing step necessary when using cyclone; Can reach a final fineness of < 40 µm Use of an ultrasonic cleaner helps to wash the sieves Successive grindings through a sieve at the same mesh size results in a higher yield of small material	Can generally reach a final fineness of < 300 µm	Minimum quantity/volume of material necessary	Fit for small quantities	/	Challenging handling, minimum quantity/volume of material necessary	In order to facilitate the work, several blades and bowls are needed

* 7 is because there are also two ZM100 versions which is an older version of the ZM200 grinder

Annex III

Flow chart of the successive steps of the sample preparation procedure for particulate material



Annex IV

Mathematical formula which might be very helpful in GMO detection for determination of the laboratory sample size when testing kernels

When a lot of kernels is analysed, it is impossible to state that it does not contain any transgenic kernel because this can only be stated if all kernels have been analysed. However what can be provided is a probability level that the lot does not exceed a certain content of transgenic kernels.

When a lot is spiked at a certain level with transgenic kernels into a lot of negative kernels, a certain amount of transgenic kernels will be added to what is considered as an amount of negative material. This negative material should have been checked to not exceed a level of transgenic material that is 20 times less the amount of the spiking level and this at a probability of 95 %. To determine this, the following formula is of great help:

$$\alpha = (1 - p)^N$$

Where:

α is the complement of the probability level i.e. if $\alpha = 0.05$, this corresponds to a probability level of 95 % (in fact $1-\alpha$).

p is the amount of transgenic material not to be exceeded in the negative lot and expressed as a ratio

N is the number of kernels to be analysed.

So, if one wants to spike a maize lot at 0.1 % of transgenic maize, the lot of non-transgenic maize should first be analysed in order to check that it does not already exceed a concentration of $0.1 \% / 20 = 0.005 \%$ of that maize in what is the negative material. To check this, one should analyse N kernels:

$$N = \frac{\text{Log } \alpha}{\log(1 - p)}$$

This will deliver that approximately 60 000 kernels (in fact exactly 59 913 kernels) should be analysed to check that they are all negative.

Other examples of application of the formula:

If one wants to check that a lot contains less than 0.01 % of transgenic linseed in a linseed batch at a probability of 95 %, the numbers of kernels to be analysed is around 30 000 (the exact outcome of the formula is: 29 956).

The formula might also be of interest for operators to know what amount of kernels to check if for instance one fears that a soybean or a maize lot might be contaminated by linseed and to check if decontamination was done correctly. Let us consider that the background contamination level of linseed is 0.005 %. What is then the number of contaminating linseeds that should not be exceeded in the laboratory sample of maize or soybean in order to avoid its detection in 99 % of cases? The formula provides the answer which is 918 kernels, so close to 1 000 kernels which in weight will be close to 6 g according to **Table 2**.

Annex V

List of food matrices that do not need homogenisation or grinding

The list provided in **Table 9** is based on an official Italian document (Ministero della Salute, 2012) which makes a classification between food products that are considered as homogeneous and those that aren't. Moreover within the non-homogeneous products it also makes the distinction between the ones needing grinding and those that do not. Mind that for this classification what is aimed to look at is important because energy bars for instance are ranked in homogeneous products (but only when looking for genetically modified soybean or maize not for something else). Moreover it is not because a product is homogeneous that there is no need for a comminution step. Bread is considered as homogeneous but to perform the extraction comminution is required.

Table 9 — List of food matrices that don't need homogenisation or grinding (derived from Ministero della Salute, 2012).

Main Food type	Product that can be considered as homogeneous (which does not mean that comminution is not necessary, it might be required for the DNA extraction step)	Product that cannot be considered as homogeneous	
		Product requiring only homogenisation	Product requiring homogenisation and grinding
Cereal corn and rice products and bakery products	Noodles, dumplings, etc. of corn and rice	Maize and rice flour or mixes	Maize kernels, maize for popcorn, sweet maize, rice kernels
			Corn flakes, muesli, pop corn
		Bread, cakes, pastry biscuits	
Edible vegetables and derived products			Canned sweet corn and soybeans, even presented in mixed salads with baby corn
Roots and tubers		Potato starch, potato flour and flakes of potato	Potato, cooked potatoes
Oil seeds		Soybean flour	Soybean kernels, linseeds, canola seeds, cotton seeds
Fruit	Papaya juice		Papaya (if coming from one same fruit, it is genetically homogenous — however avoid taking the seeds)
Vegetable drinks and products derived from vegetable milk	Soy milk and drink, soy cheese, béchamel, soy yoghurt, tofu, soy fluid		
Baby food	Soy milk vegetable baby food		
Miscellaneous edible preparations	Ice creams and sorbets, desserts, puddings, creams, custards, products coming from extrusion processes (tortilla chips), energy bars (for this latter ones, only if detection is addressing genetically modified soybean or maize)		
Meat imitates	Textured soy protein products		

Annex VI

The paper cone riffle splitter (for information only)

A device presented by Gerlach et al. (2002), called paper cone riffle splitter (see **Figure 10**) might be compatible with sample preparation for GMO detection because the risk of cross-contamination could be avoided by replacing the filter for each sample. However, this device does not seem to be commercially available. Moreover in the configuration illustrated in **Figure 10**, there is also a problem of loss of sample to be solved. This device is cited here just because it integrates improvements which would make its use feasible for GMO detection but in practice the device does not seem to be available.

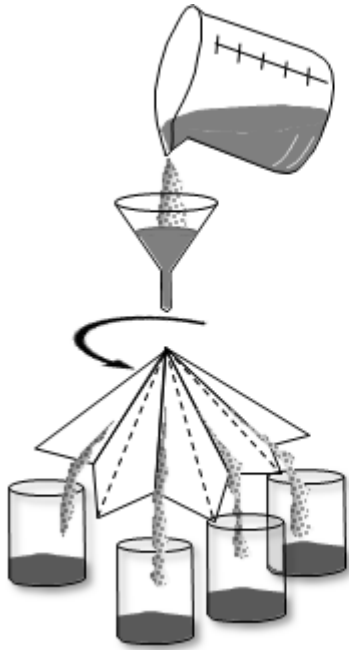


Figure 10 — The paper cone filter riffle splitter (Figure adapted from Gerlach et al., 2002).

Annex VII

Replication experiment

To perform this replication experiment at least 10 replicates are to be considered and this should be carried out as indicated in **Table 10**. In this table three levels have been considered but it is possible to extend it to a fourth or a fifth level if required. It is then possible to calculate the total relative subsampling variation but also the contribution of each step to the whole variability. The three steps considered in **Table 10** could for instance be:

- A) a mass reduction step + the grinding of the resulting subsample
- B) the uptake of the test portion + the DNA extraction
- C) the PCR analysis

Step A should be done in 10 replicates and the total analysis with steps B and C should be performed on each of the replicates (from A100 to A10 00). This will lead to ten results (C111, C200 to C10 00) from which the relative standard deviation (corresponding to RSV_1 in **Table 10**) can be calculated.

Step B will also be submitted to a special replicate analysis on the material of step A100. This will also generate ten results (C111, C120 to C1 10 0) from which the relative standard deviation (corresponding to RSV_2 in **Table 10**) can be calculated.

Step C will then lastly also be submitted to a separate replicate analysis but limited to the material generated by step B110.

Table 10 –The replicate analysis to distinguish the source of variability of three successive steps (A, B and C).

<p>1) from level A to C</p> <p>A: A1.0.0 A2.0.0 A3.0.0 A4.0.0 A5.0.0 A6.0.0 A7.0.0 A8.0.0 A9.0.0 A10.0.0 B: B1.1.0 B2.0.0 B3.0.0 B4.0.0 B5.0.0 B6.0.0 B7.0.0 B8.0.0 B9.0.0 B10.0.0 C: C1.1.1 C2.0.0 C3.0.0 C4.0.0 C5.0.0 C6.0.0 C7.0.0 C8.0.0 C9.0.0 C10.0.0 ⇒ RSV_1</p> <p>RSV_1 provides the global subsampling variability from step A to C</p>
<p>2) from level B to C</p> <p>B: B1.1.0 B1.2.0 B1.3.0 B1.4.0 B1.5.0 B1.6.0 B1.7.0 B1.8.0 B1.9.0 B1.10.0 C: C1.1.1 C1.2.0 C1.3.0 C1.4.0 C1.5.0 C1.6.0 C1.7.0 C1.8.0 C1.9.0 C1.10.0 ⇒ RSV_2</p> <p>RSV_2 provides the subsampling variability from step B to C</p>
<p>3) level C</p> <p>C: C1.1.1 C1.1.2 C1.1.3 C1.1.4 C1.1.5 C1.1.6 C1.1.7 C1.1.8 C1.1.9 C1.1.10 ⇒ RSV_3</p> <p>RSV_3 provides the variability of level C</p>
<p>To calculate the specific contribution of variability of level B:</p> $RSV_B = \sqrt{(RSV_2)^2 - (RSV_3)^2}$ <p>To calculate the specific contribution of variability of level A:</p> $RSV_A = \sqrt{(RSV_1)^2 - (RSV_2)^2} = \sqrt{(RSV_1)^2 - (RSV_B)^2 - (RSV_3)^2}$

For sampling the whole variability expressed in terms of relative standard variation should not exceed 33 %. As we are here in fact at subsampling level it should even be less and therefore a figure of 25 % seems appropriate as value that should not be exceeded. However it is not at all impossible that in some cases the variability cannot be below that figure.

Europe Direct is a service to help you find answers to your questions about the European Union
Freephone number (*): 00 800 6 7 8 9 10 11

(*) Certain mobile telephone operators do not allow access to 00 800 numbers or these calls may be billed.

A great deal of additional information on the European Union is available on the Internet.
It can be accessed through the Europa server <http://europa.eu/>.

How to obtain EU publications

Our priced publications are available from EU Bookshop (<http://bookshop.europa.eu>),
where you can place an order with the sales agent of your choice.

The Publications Office has a worldwide network of sales agents.
You can obtain their contact details by sending a fax to (352) 29 29-42758.

European Commission
EUR 27021 EN – Joint Research Centre – Institute for Health and Consumer Protection

Title: Guidelines for sample preparation procedures in GMO analysis

Author(s): Prepared by the ENGL ad hoc working group on “sample preparation procedures”

Luxembourg: Publications Office of the European Union

2014 – 38 pp. – 21.0 x 29.7 cm

EUR – Scientific and Technical Research series – ISSN 1831-9424 (online), ISSN 1018-5593 (print)

ISBN 978-92-79-44704-4 (PDF)

ISBN 978-92-79-44705-1 (print)

doi:10.2788/738570

JRC Mission

As the Commission's in-house science service, the Joint Research Centre's mission is to provide EU policies with independent, evidence-based scientific and technical support throughout the whole policy cycle.

Working in close cooperation with policy Directorates-General, the JRC addresses key societal challenges while stimulating innovation through developing new methods, tools and standards, and sharing its know-how with the Member States, the scientific community and international partners.

*Serving society
Stimulating innovation
Supporting legislation*

