

Aggiornamenti sui lavori del Working Group ENGL “Sample preparation procedure”

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Guidelines for sample preparation procedures in GMO analysis

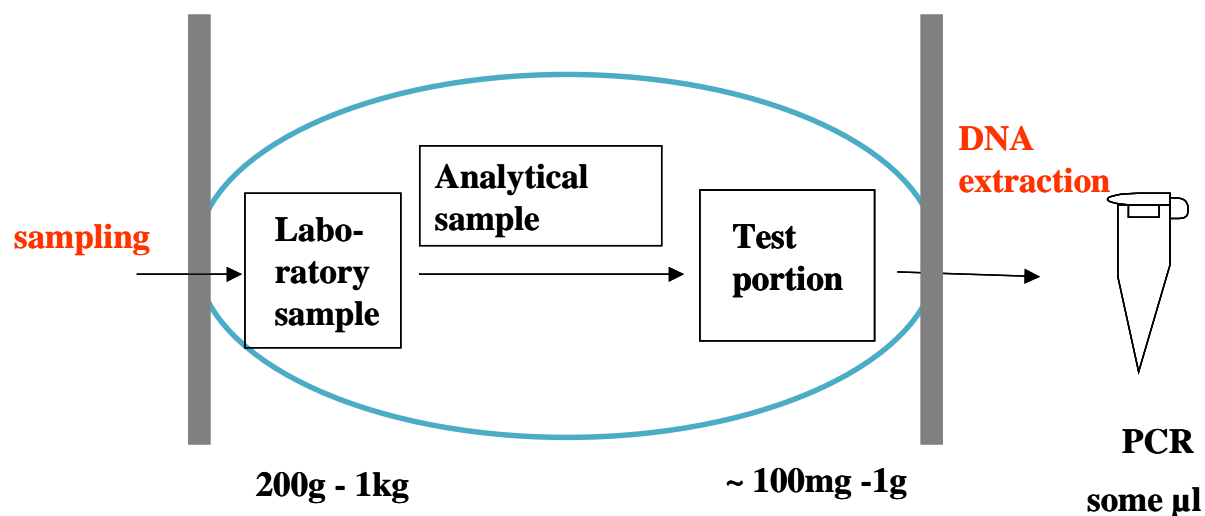
Prepared by the

ENGL *ad hoc* working group on “sample preparation procedures”

Final version after comments of SC and WG members (version 12)

Guidelines for sample preparation procedure in GMO analysis

Precedure utilizzate per la riduzione (sub-sampling) ed omogeneizzazione a partire dal campione di laboratorio fino ad ottenere l'aliquota da saggio



Working Group on Sample Preparation Procedure

Scopo del mandato

- Ridurre l'errore associato a questa fase
 - Granulometria ottimale per incrementare la rappresentatività e la resa in DNA
 - Impiego di procedure standardizzate basate sulla TOS
 - Pros and cons correlati all'impiego di differenti sistemi di omogeneizzazione/macinazione.
 - Contaminazione crociata.
- Sicurezza degli operatori ed adeguatezza delle aree di lavoro
- Validazione delle procedure (controllo di qualità)
- Identificare punti critici

Guidelines for sample preparation procedure in GMO analysis

***'Animal feeding stuffs - Guidelines for
sample preparation' ISO/FDIS 6498***

**Rivisto ed ampliato per l'applicazione al
settore analitico OGM**

***CEN/TS 15568:2006 (E) ISO 24276:2006
Rec. (EC) No 787/2004
Regulation (EC) No 619/2011***

Guidelines for sample preparation in GMO analysis

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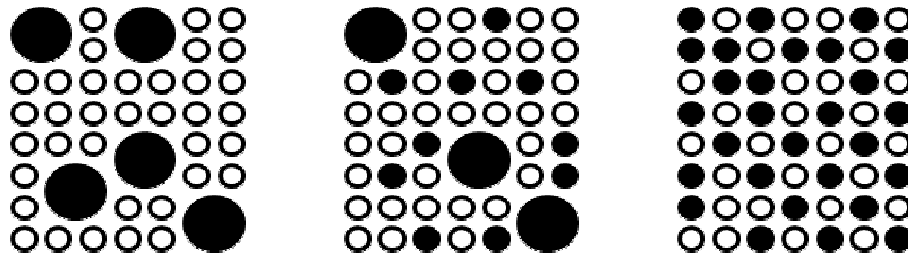
4.General considerations linked to the theory of sampling

Errore legato alle caratteristiche del campione da analizzare

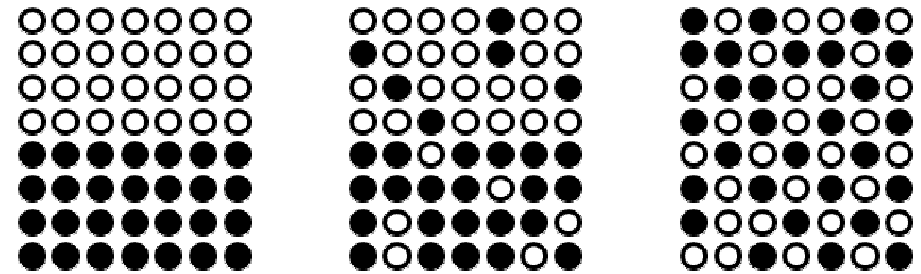
Errore legato alla riduzione del campione (sub-sampling)

Errore legato alle caratteristiche del campione da analizzare

Disomogeneità costituzionale



Distribuzione eterogenea



Constitutional heterogeneity

Distributional heterogeneity

macinazione

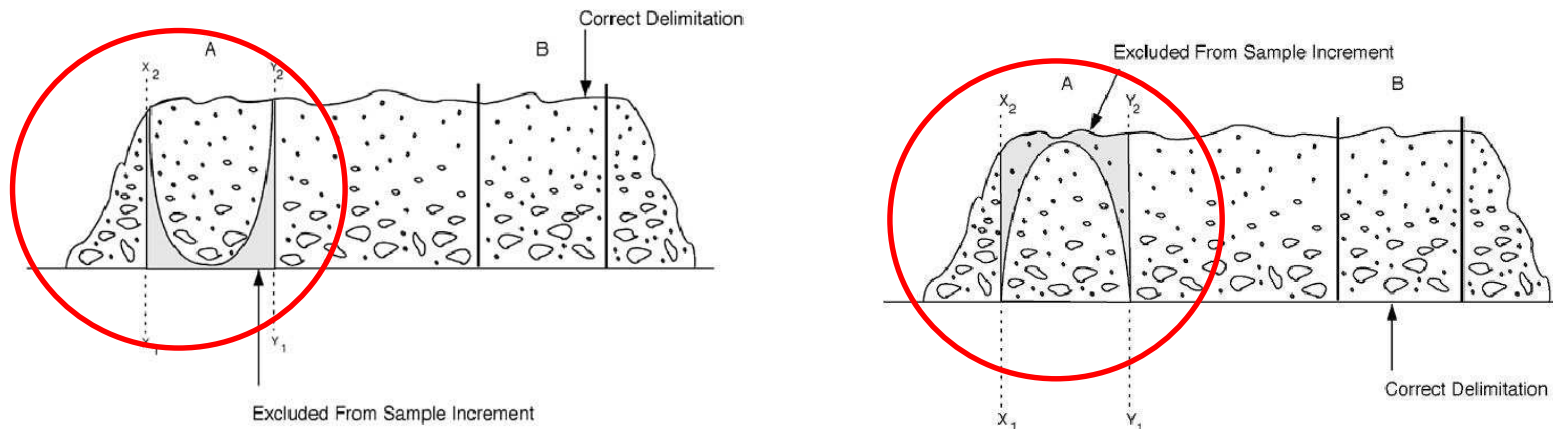


mescolamento

Errore legato alla riduzione del campione (sub-sampling)

Derivante dalle procedure utilizzate per:

- Selezionare e prelevare gli incrementi (increment delineation and extraction)



Use of **inappropriate extraction tools** can result in an **incorrect delineation** of the increment that will lead to over-representation or under-representation of some fractions present in the sample

Subsampling operations

Una riduzione dell'errore può essere ottenuta utilizzando in modo corretto le seguenti procedure:

Composite subsampling (or incrementing)

Comminution (particle size reduction)

Mixing/blending

Limitano gli errori legati alle caratteristiche del campione da analizzare.

Representative mass reduction

Utilizzo di metodi di riduzione del campione idonei ad ottenere un campionamento rappresentativo

5 Attrezzature

Incorrect Design

Spatula



Flat without edges:
material segregates
when falling off
each side

Scoop



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

Shovel



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

Correct Design

Spatula



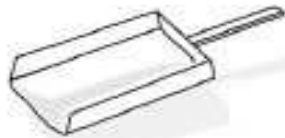
Square edges prevent
material from falling
off each side

Scoop



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

Shovel



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

Sampling tools

Spoons, spatulas and shovels

To avoid the increment delimitation errors it is advised that spoons, spatulas and shovels should be square-edged

Equipment for particle size reduction and mixing

Grinders or blenders

Cleaning tools

Brushes for cleaning grinders, etc

Compressed air blower for cleaning

Vacuum cleaner

Ultrasound bath (e.g. useful for cleaning sieves)

6. Environment and rooms

6.1 Room

According to ISO Standard 24276:2006,

6.2 Cleaning and decontaminating reagents

List of reagents to be used

7. Safety precautions

- dust ventilation system has to be operated during dust generation procedures.
- vacuum cleaner can for instance be used to minimize dust in the hood area and the working area.
- caref 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

8.1 Laboratory sample check

8.1.1 Check of laboratory sample constitution

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 3000 kernels (see table below for mass equivalent of 1000 kernels)
Commodity grains	Mass equivalent of 10000 grains (see table below for mass equivalent of 1000 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

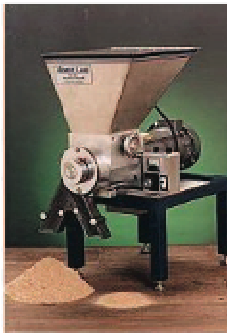
Annex V Lista delle matrici e livello e di omogeneità

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		

8.2 Mass reduction

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**



8.3.2 Requirements regarding the choice of size reduction equipment

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

Annex II

Examples of grinders used by laboratories involved in GMO detection (1)

Brand (of the grinder)	Retsch	Retsch	Vorwerk
Type	ZM200	Grindomix GM200	Thermomix 21
Maximal capacity that can be ground at once (in grams unless other unit used)	Up to 300 ml with standard cassette (4500 ml with cyclone)	Up to 700 ml	2000 (depending on mass and volume)
Number of laboratories using this equipment	5 (7)*	3	1
Is there any integrated system for size control of particles?	Yes (due to sieve)	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 min (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 min (pot, lid, blade) Time reduction when using extra pots/blades and dishwasher
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)
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	Can generally reach a final fineness of < 300 µm	Minimum quantity/volume of material necessary

Annex II

Examples of grinders used by laboratories involved in GMO detection (2)

Brand (of the grinder)	IKA	IKA	Maxi Grinder	Waring
Type	A11 basic	M20	Solo	Blender, 1, 2 and 4l capacities
Maximal capacity that can be ground at once (in grams unless other unit used)	250 (depending on mass and volume)	500 (depending on mass and volume)	2000 (depending on mass and volume)	From 200 to 2000 according to the capacity
Number of laboratories using this equipment	1	1	1	1
Is there any integrated system for size control of particles?	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?	Simple 3-5 min	Medium 5-10 min	Simple 3-5 min Time reduction when using extra containers/blades and dishwasher	Medium 10 min
Are there matrices that do not fit?	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)	Fit for small quantities	/	Challenging handling, minimum quantity/volume of material necessary	In order to facilitate the work, several blades and bowls are needed

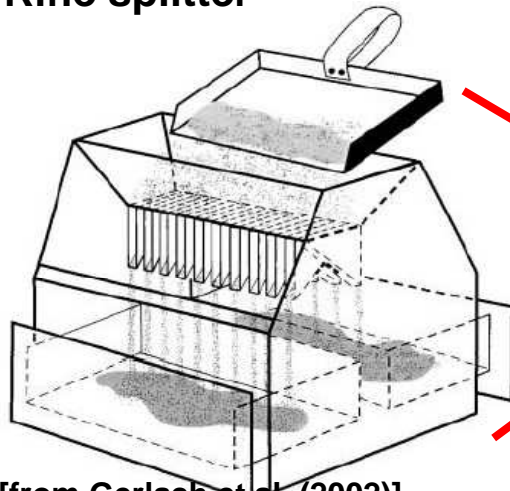
8.2 Mass reduction

8.2.2 Subsampling procedures

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

Rifle splitter

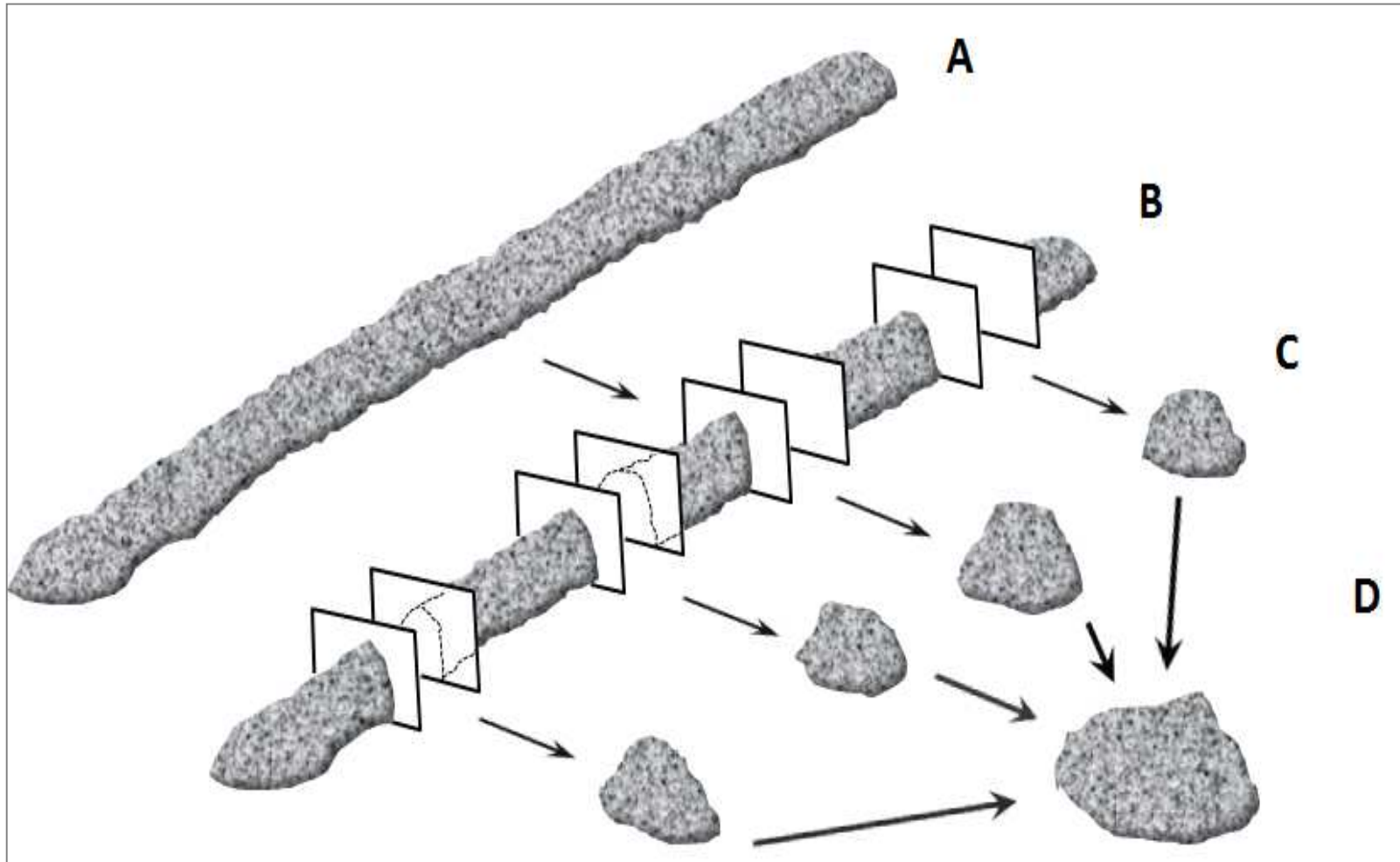


[from Gerlach et al. (2002)]



8.2 Mass reduction

8.2.2.3 long pile method



8.3 Particle size reduction

8.3.1 General methods

8.3.2 Requirements regarding the choice of size reduction equipment

8.3.3 Maintaining integrity of the laboratory sample

Esempi pratici

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;

8.3.2 Requirements regarding the choice of size reduction equipment

Percentage of recovery according to the granulometry

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 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 noodles	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

Janssen, E. (2013). DNA extraction from fresh papayas and rice products. NRL-GMO practical workshop “sample preparation – DNA extraction from different matrices”, Brussels, 06 June 2013. Available on : https://intralab.favv-afscs.be/labo/formations/presentations/documents/2-NRL-GMOworkshop_06062013_E.Janssens.pdf

8.3 Particle size reduction

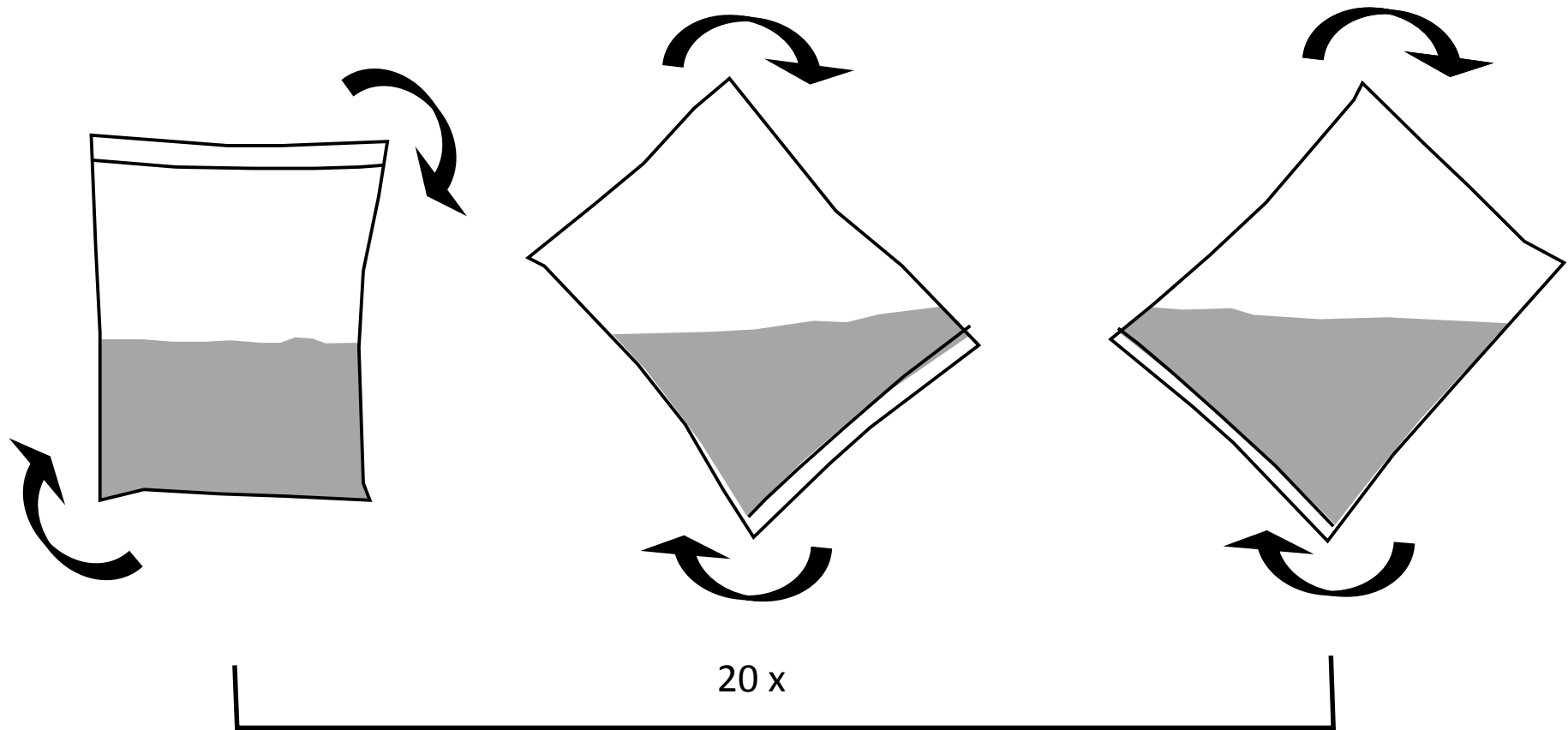
8.3.4 Mixing techniques

Esempi pratici

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.**

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.

8.3.4.2 Homogenisation by mixing with the plastic bag technique



Special sample preparation procedures

Campioni viscosi

Il riscaldamento a 40-60°C migliora l'omogeneità e di conseguenza la rappresentatività dei campioni viscosi (es. miele e lecitine).

Campioni liquidi

I campioni liquidi sono generalmente considerati omogenei (Ministero della Salute 2012). Possono però esserci delle eccezioni: per l'olio è consigliato il metodo descritto da Costa (Costa et al.2010) che prevede una centrifugazione a circa 18.000 g per 30 min, seguita da estrazione su pellet.

Tessuti di origine vegetale

Due differenti procedure sono applicabili: l'uso dell'azoto liquido seguito da frantumazione del materiale con un pestello; congelamento (più di un giorno) seguito da macinazione del campione.

Matrici semisolide

Per alcune matrici semisolide con meno dell'85% di materia secca (es. foraggio, pane fresco) è necessario disidratare parzialmente prima della macinazione, utilizzando un forno con ricircolo di aria o un microonde. E' importante mantenere la temperatura sotto i 55-60 °C per ridurre al minimo la degradazione del DNA.

8.6 Test portion

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

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. tumbler mixer) can be used for this purpose.



The test portion **uptake** is generally done using **grab sampling**, since care has been taken to prepare a homogeneous test sample: grab sampling at this level is generally deemed acceptable.

8.6 Test portion

8.6.2 Minimum mass of test portion

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) Maximum particle size (d)	25%	20 %	10 %	5 %	2 %	1 %
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

9 Performance tests (quality control)

9.1 Performance test for particle size reduction (grinding)

9.1.1 Grinding quality

centrifugal grinders with sieves at defined mesh sizes several regular performance tests (e.g. **on an annual basis**) can be designed: the ground product should consist of at least 80 % of particles below half of the mesh size;. A good matrix for these performance tests is **wheat or rice**.

9.1.2 Carryover

9.1.2.1. Negative sample test

analysing a negative sample following a positive sample with preferably a rather high content of GM material (> 5 %). The cleaning process will of course have been carried out in between both samples.

A crop for which no commercial GM varieties currently exist (e.g. wheat) could be used as an alternative for a negative sample.

9 Performance tests (quality control)

9.4 Representativity of the test portion

The test portion used in GMO analysis is **always small** it is important to check that its **size** combined with the **grinding technique** ensures a sufficient **representativity of the sample within the test portion**.

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

A way to do this is to check on kernels if a level close to the limit of detection can be reliably detected during replicate analyses on the ground material: **e.g. analyse at least 6 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 during grinding the **yielded ground material should be smaller**.

The LOD of the whole analytical process

Grind **three different samples**, each consisting of **one GM kernel in n kernels of the same non GM species**. Or different species of similar size (e.g. maize and soy, tomato and rapeseed, wheat and barley).

From each grinding, **perform 6 DNA extractions, 18 extractions** in total.

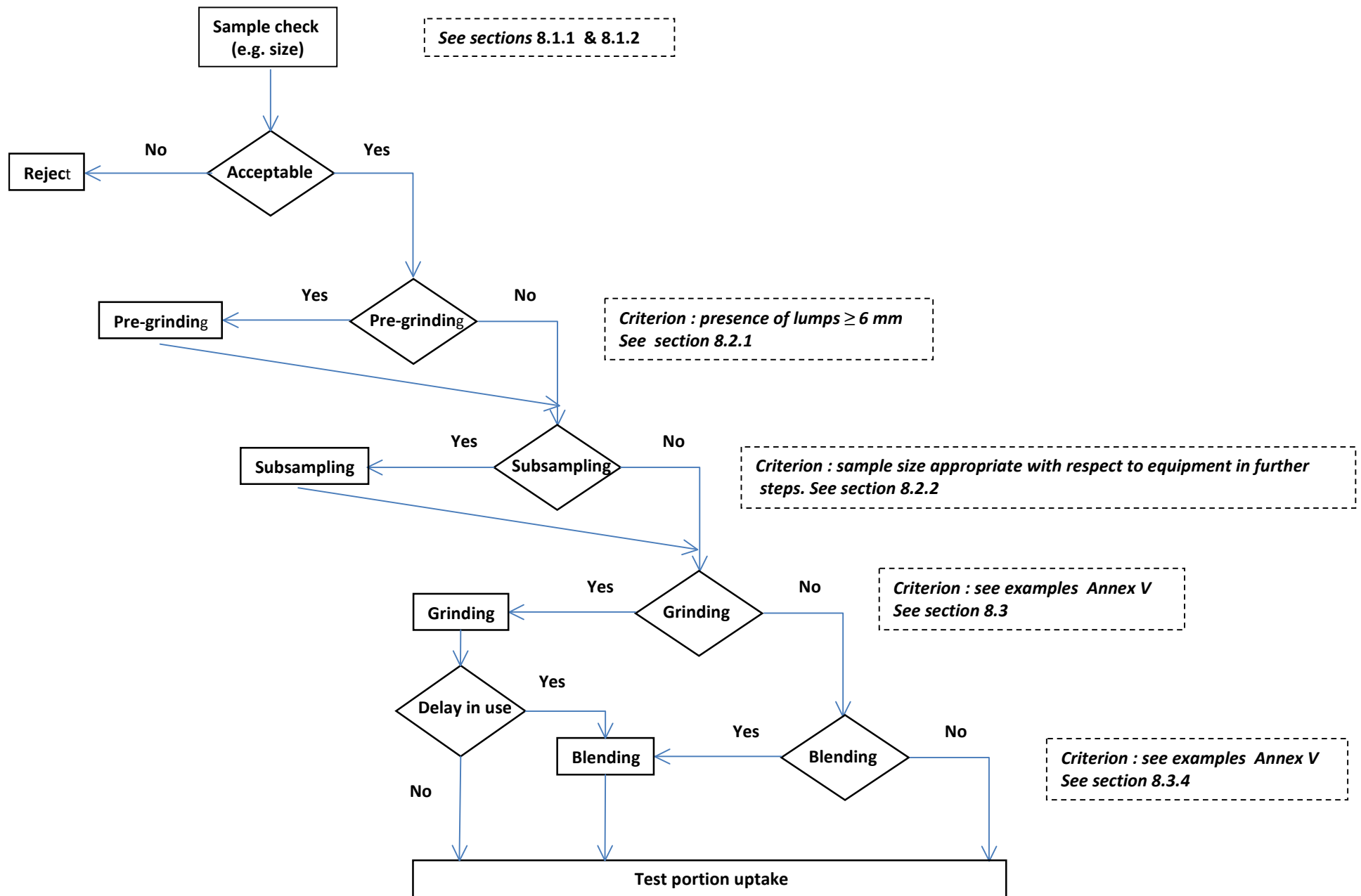
Then perform a PCR (targeting either the GM event or the other plant species) on the 18 extractions. If they are **all positive** then $1/n$ will be the limit of detection of the whole analytical process.



All PCR results positive

Annex III

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



10 Identified gaps and conclusions

➤ Procedure Operative Standard applicabili direttamente dai tecnici



➤ Lista di situazioni possibili con un'analisi dei possibili errori che devono essere evitati rivolta ai responsabili di Laboratorio



➤ Procedura riportata nell' **annex VII** può fornire dati utili per valutare l'incertezza associata alle differenti fasi della preparazione del campione per matrici non considerate nel documento

10 Identified gaps and conclusions

➤ Suggestimento di effettuare 10 replicati è un compromesso non supportato da evidenze scientifiche



➤ Sarebbe necessario avere maggiori dati sperimentali (non previsti dal mandato) per supportare le indicazioni che si basano sui dati disponibili

➤ Campioni di 3000 or 10000 semi non sono facili da gestire patata ???



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Grazie per l'attenzione