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BSI Standards Publication

Ambient air — Monitoring the effects of genetically modified organisms (GMO) — Pollen monitoring

Part 1: Technical pollen sampling using pollen mass filter (PMF) and Sigma-2-sampler

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National foreword

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Ambient air - Monitoring the effects of genetically modified organisms (GMO) - Pollen monitoring - Part 1: Technical pollen sampling using pollen mass filter (PMF) and Sigma-2-sampler

Air ambiant - Surveillance des effets d'organismes génétiquement modifiés (OGM) - Surveillance du pollen - Partie 1 : Échantillonnage technique du pollen à l'aide d'un filtre de masse à pollen (PMF) et d'un échantillonneur Sigma-2

Außenluft - Monitoring der Wirkungen von gentechnisch veränderten Organismen (GVO) - Pollenmonitoring - Teil 1: Technische Pollensammlung mit Pollenmassenfilter (PMF) und Sigma-2-Sammler

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European foreword

This document (CEN/TS 16817-1:2015) has been prepared by Technical Committee CEN/TC 264 “Air quality”, the secretariat of which is held by DIN.

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CEN/TS 16817, *Ambient air — Monitoring the effects of genetically modified organisms (GMO) — Pollen monitoring,* is composed of the following parts:

— *Part 1: Technical pollen sampling using pollen mass filter (PMF) and Sigma-2-sampler* [the present document];

— Part 2: Biological pollen sampling using bee colonies.

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Introduction

The European Parliament and the European Council require an environmental risk assessment and a post-marketing monitoring for any GMO released to the environment [5; 6]. This had to be implied in national law in any member state of the EC by date.

Pollen dispersal plays a significant role in the dissemination of genetically modified organisms (GMO). A procedure is described for GMO monitoring that enables quantification and documentation of GMO input and spread through pollen in a nationwide monitoring network which represents natural landscapes. Technical and biological pollen sampling (the present Technical Specification and CEN/TS 16817-2) and molecular biological analysis methods (polymerase chain reaction (PCR) for DNA; Enzyme-linked immunosorbent assay (ELISA) for proteins) are used for the detection of GMO input.

It is reasonable to use both technical and biological sampling of pollen, thus they supplement each other in manifold ways. The technical sampling (i.e. the present document) is conducted with stationary point-samplers. They give a record of pollen input at the sample site that correlates with the prevailing wind direction and relative position to the surrounding pollen sources. Bee colonies actively roam an area and are therefore area related samplers. Further, pollen sampling depends here on the collection activity of the bees and the availability of pollen sources within the roaming area according to the bees' preferences and supply of melliferous plants [32].

Presently known pollen traps are only partially suited for GMO monitoring, since they can neither be standardized nor is the instrumentation designed for exposure times that are suitable for this purpose. Another limitation of commonly used pollen samplers is the requirement for a power supply, e.g. as for the Hirst type trap. The use of these instruments is therefore restricted to a limited exposure area.

For these reasons, a new type of passive pollen sampler, the pollen mass filter (PMF), was developed. The PMF is used either in combination with the Sigma-2 passive sampler or solely.

The present Technical Specification is largely based on German VDI/Guideline 4330 Part 3 [31].

1 Scope

This Technical Specification describes a procedure for the use of the passive samplers Sigma-2 and PMF to sample airborne pollen. Both are designed to sample coarse aerosol particles. Collected samples are used to analyse pollen input with regard to pollen type and amount, and input of transgenic pollen. The Sigma-2 passive sampler here provides a standardized sampling method for direct microscopic pollen analysis and quantifying the input of airborne pollen at the site. The PMF yields sufficient amounts of pollen to additionally carry out molecular-biological diagnostics for detection of GMO.

Essential background information on performing GMO monitoring is given in VDI/Guideline 4330 Part 1 [4], which is based on an integrated assessment of temporal and spatial variation of GMO cultivation (sources of GMO), the exposure in the environment and biological/ecological effects. Ideally, the pollen sampling using technical samplers for GMO monitoring should be undertaken in combination with the biological collection of pollen by bees (CEN/TS 16817-2).

The application of technical passive samplers and the use of honey bee colonies as active biological collectors complement each other in a manifold way when monitoring the exposure to GMO pollen. Technical samplers provide results regarding the pollen input at the sampling site in a representative way, whereas with biological sampling by honey bee colonies, pollen from flowering plants in the area is collected according to the bees' collection activity. Thus, this method represents GMO exposure to roaming insects. By combining the two sampling methods these two main principles of exposure are represented. Furthermore, a broad range of pollen species is covered.

The sample design depends on the intended sampling objective. Some examples are given in 6.2.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

VDI 2119:2013-06[1)], *Ambient air measurements — Sampling of atmospheric particles > 2,5 µm on an acceptor surface using the Sigma-2 passive sampler — Characterisation by optical microscopy and calculation of number settling rate and mass concentration*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
acceptor surface
natural or manmade collection surface for airborne particles

3.2
concentration
number concentration
number of particles per unit air volume; here number of pollen per m^3 air

3.3
deposition
pollen deposition
deposition of atmospheric particles; here pollen on an acceptor surface

1) For application of the Sigma-2.

3.4
dispersal
pollen dispersal
spread of pollen from the flower/field into the surrounding environment by wind drift

3.5
event
<genetics> unique DNA recombination event that took place in one plant cell, which was then used to generate entire transgenic plants

3.6
flux
horizontal flux
number of particles (here pollen) that are drifted horizontally per wind

3.7
genetically modified organism
GMO
organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination

[SOURCE: Directive 2001/18/EC [5], modified — The content of the definition was changed.]

3.8
monitoring
environmental monitoring
characterizing the state and quality of the environment and its changes by measurements/observations in regard to defined objectives

3.9
pollen
male gametophyte of the flowering plant

3.10
pollen type
species
class of pollen being distinguished by microscopic means on species, family or other order level

3.11
sampler
device for sampling here of pollen

3.12
sampling
pollen sampling
collection of particles, here pollen by technical or biological means

3.13
sedimentation
directed particle movement by gravity (here pollen in the air), which consists in a vertical flux towards the ground

4 Basic principle of the procedure

For the technical pollen sampling, two passive samplers are used, the PMF and Sigma-2 passive sampler, either in combination or the PMF solely:

The Sigma-2 passive sampler is designed for determining the pollen deposition rate (dry deposition by sedimentation). Wind-dispersed pollen grains enter the interior through the laterally shifted slits of the sampler. The pollen are deposited on an adhesive tray as acceptor (tape, foil, slide) at the bottom of the sampler. Thus, the deposition takes place in the turbulence-depleted interior of the sampler which provides protection from wind and rain. Pollen adhering to the adhesive tray are directly analysed with regard to pollen type and counts by means of light microscopy. For the purpose of GMO monitoring, an exposure time in the range of four weeks is recommended to be able to cover the main flowering period of the target plant species with as few sampling periods as possible (the rationale for this is given in 6.5). The microscopic single-particle analysis yields an average pollen deposition rate for the respective pollen species and time period. Summing up the deposition rates of all sampling periods in the season yields the total pollen deposition per season/year as target parameter.

The pollen mass filter (PMF) exhibits a 10 times to 100 times higher sampling efficiency, so that pollen samples can be analysed both microscopically to quantify pollen input and further on, with regard to possible GMO input by using molecular-biological based methods (e.g. PCR for DNA, ELISA for proteins/toxins). The PMF consists of a layered hollow filter that is constructed in such a way as to let the air pass through nearly unopposed. However, coarse aerosol particles bigger than 10 µm, such as pollen, are retained. A laterally mounted collection flask is used for collecting rainwater. For the PMF, an exposure time of four weeks is recommended (see 6.5) so that only a few samples are needed to cover the relevant flowering period. In order to cover a complete blooming period of one or more target plant species a respective number of exposure (sampling) periods lasting four weeks each can be carried out.

The Sigma-2 passive sampler collects aerosol particles bigger than 1 µm covering the size range of most pollen and fungal spores. Its sampling efficiency reaches its limitation towards bigger and heavier aerosol particles over 60 µm diameter, like e.g. maize pollen. In such cases, the evaluation of pollen deposition shall be based on the PMF solely.

Field experiments have shown that the method is well suited for environmental monitoring of GMO [14; 17; 20].

5 Sampling

5.1 Instruments and materials

5.1.1 General

The combined sampling equipment consisting of a Sigma-2 passive sampler and PMF is described in Figure 1. For some tasks the PMF sampler is used solely, e.g. for maize pollen, and/or when it is necessary to increase the amount of sampled pollen at a site within a certain period (see 10.5.2, e.g. for keeping detection limits for PCR-analysis of pollen DNA). For such tasks, stacked versions of the PMF sampler with more than one PMF-unit per sampler are additionally available as shown in Figure 2. The complete sampling equipment is available.[2) 3)]

2) TIEM technic GbR, Hohenzollernstr. 20, 44135 Dortmund. Samplers are manufactured by the supplier mentioned above. It is an example of a suitable product. This information is given for the convenience of users of this European Technical Specification and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

3) Breitfuß Messtechnik GmbH, Danziger Str. 20, 27243 Harpstedt. Samplers are manufactured by the supplier mentioned above. It is an example of a suitable product. This information is given for the convenience of users of this European Technical

5.1.2 Sigma-2 passive sampler

The Sigma-2 device is a passive sampler for coarse atmospheric particles as described in VDI/Guideline 2119. It consists of a cylindrical sedimentation chamber with a protective hood on top as inlet.

As acceptor surface for particle deposition, conventional microscopic slides are recommended (76 mm × 26 mm) with two quadratic acceptor fields (18 mm × 18 mm) coated with weather-proof adhesive, such as polymeric acrylic ester. The slides are attached to the base with an adapter. Alternatively adhesive acrylic foil (60 mm × 60 mm), also coated with weather-proof adhesive, on special adapters can be used, too.

Prepared adhesive foils and slides for sample handling and suitable shipping cans are available from the supplier mentioned above. It is recommended to use slides with marked acceptor fields (frames with plotted scales, dots or lines) for facilitating the microscopic pollen analysis. In this TS the handling is described for slides only.[4)]

5.1.3 Pollen mass filter PMF

The PMF consists of:

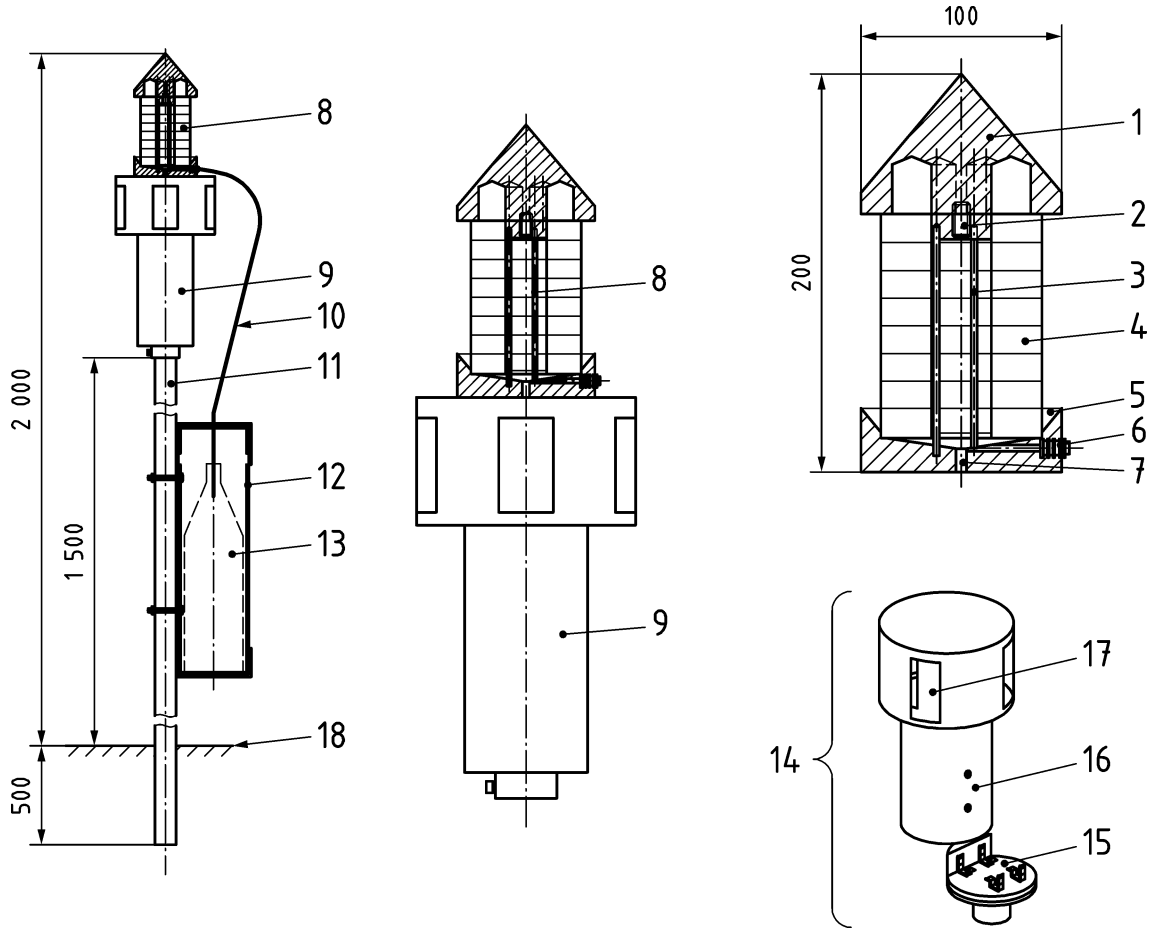
— filter cladding (depth filter) consisting of a stack of eight filtering discs;

— filter holder conical top, base with distance rods and quick-connect tube coupling;

— collection flask, sheath, connecting tube, floor stand pole, length 2 m, diameter e.g. 34 mm.

Specification and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

4) For the handling of foil as acceptor, see Guideline VDI 2119.



Key

1 conical top
2 screwed joint for removing filter discs
3 distance rods
4 filter cladding (consisting of eight stacked filter discs)
5 base with conical outlet
6 quick-connect tube coupling’
7 screw joint to Simga-2 passive sampler
8 complete PMF sampling unit
9 Sigma-2 passive sampler
10 connecting sample tube
11 pole, length 2 m
12 sheath for collection flask
13 collection flask
14 Sigma-2 passive sampler opened
15 base with acceptor surface and adapter
16 sedimentation cylinder
17 inlet (top cover with slits)
18 ground

Figure 1 — Detailed views of the complete sampling equipment [[17]; VDI 2119:2013-06]

a) PMF/Sigma-2 passive sampler **b) PMF-mono** **c) PMF-duo** **d) PMF-trio** **e) PMF-quattro**

Figure 2 — Types of technical sampler [Source: TIEM technic GbR]

5.2 Technical implementation

The Sigma-2 passive sampler provides a suitable method for sampling airborne particles including pollen. This instrument consists of a cylindrical sedimentation chamber which is covered by a cylindrical hood. Both parts have notches enabling air exchange between the interior of the sedimentation chamber and ambient air. Inside the chamber with a volume of calmed air particles suspended in the air sediment onto a transparent adhesive foil or slide that can be removed for subsequent microscopic analysis. Under the microscope, particles including pollen can be identified and counted. The average pollen deposition rate for the exposure time is calculated from the pollen count determined on a defined area on the adhesive carrier exposed over a defined time (see 9.2.1). However, within the conventional exposure time the amount of pollen collected by the Sigma-2 passive sampler is insufficient to carry out unambiguous pollen DNA analyses using PCR techniques for the detection of specific gene sequences such as transgenes.

In order to collect sufficient amounts of pollen for molecular-biological analyses the passive sampler PMF was developed.

The PMF consists of a filter holder with filter cladding as sampling unit, collection flask, and connecting tube. The PMF sampling unit is screwed on top of the Sigma-2 passive sampler or used solely with an adapter. The PMF sampling units can be stacked up to four units per pole.

The PMF filter cladding provides a low aerodynamic resistance and flux of ambient air through the filter. In addition, the characteristics of the filter material ensure that pollen and other particles larger than 10 µm in diameter can attach and adhere to the surface of the fibres. If it rains, some of the pollen and other particles can be washed off the surface of the fibres. Therefore, the rainwater is collected in the collection flask. The samples for downstream analyses are extracted from the filter material and from the rainwater in the collection flask using a sample preparation procedure.

— **Filter holder** (Figure 1, parts 1, 2, 3, 5, 6, and 7):

The filter holder consists of the conical top and the base part.

The conical shape of the top prevents birds from landing on it.

Three distance rods in the base part form a track for the filtering discs. The rods' length determines the height of the filter cladding and the pressure between the filtering discs.

Rain water is collected in the flanged collar of the base part, the bottom of which is conical – thus, water is drained off through the quick-connect coupling and connecting tube into the collection flask.

The conical top of the filter holder can be screwed off in order to insert the filter cladding.

Components are made up of inert materials (e.g. aluminium with an anodized surface, stainless steel).

— **Filter cladding** (Figure 1, part 4):

The filter cladding consists of eight filtering discs layered on top of each other.

The annulate filtering discs have an outer diameter of 80 mm, while the centre-hole is 30 mm in diameter. Discs are punched out from a flat 20 mm thick depth filter fleece. The fleece is made of thermally bound and progressively layered polypropylene fibres.

For the horizontal wind component the effective flux cross-section (height × width) of the filter cladding is 100 mm × 80 mm = 0,008 m^2 for all wind directions. Filter dimension and material are characterized by a low flux drag and back pressure.

— **Connecting tube** (Figure 1, part 10):

Polyamide tubes with an outer diameter of 6 mm and a wall thickness of 1 mm have proven to be effective as inert connecting tubes.

— **Collection flask** (Figure 1, part 13):

Clean 1,5-l PET collection flasks with flat, round bottoms are suitable for the average amount of rain-fall in Germany and sampling times of four weeks. In cases of extreme high rainfall during the sampling period (approx. more than 120 l/m^2), the overflow of the bottle will start. This will not affect the sampled amount of pollen though because they sediment to the bottle base. For record of the complete amount of rainfall in regions with higher precipitation, bigger flasks and container might be used or the flask might be changed intermediately. For visual inspection the collection flask should be transparent, but during sampling time it shall be protected from daylight by aluminium foil and placed into an opaque sheath (Figure 1, part 12).

— **Floor stand** (Figure 1, part 11):

All components of the pollen sampling equipment are mounted onto a floor stand: the Sigma-2 passive sampler with the PMF screwed on top of it, and the collection flask underneath, so that sample liquid can drain off easily.

A pole of 2 m in length with an outer diameter of 34 mm is suitable. It is helpful to drill an 11-mm wide hole through the pole 0,5 m from the bottom end, through which a stabilizing rod can be inserted. This facilitates sinking the floor stand 0,5 m into the ground.

6 Sampling procedure

6.1 General

For documentation purpose, a protocol about sampling and site conditions needs to be prepared (see 11.2).

6.2 Sampling design

6.2.1 General

The sample design depends on the intended monitoring objective. Some examples are given here:

6.2.2 Exposure assessment of pollen input in the vicinity of fields with genetically modified crop (gm-fields) related to a specific GMO and region

a) Potential tasks: Risk assessment and management, definition of appropriate buffer distances.

b) Examples:

1) Bt-maize cultivation in the vicinity of nature reserve areas inhabited by sensitive non-target organisms;

2) pollen input of gm-oil seed rape cultivation to neighbouring fields.

The technical pollen samplers are distributed in the vicinity of the gm-field(s) in such a way that the spatial distribution of pollen exposure and its gradients can be assessed in a representative way. The samplers are placed around the field(s) in different distances from the respective field up to further distances (reference areas), covering various wind directions. As the dispersal of pollen follows a power function in relation to distance, a nonlinear, logarithmic distance scale shall be applied. The sample design depends largely on the availability of secondary data relevant to pollen dispersal, e.g. meteorological data like wind direction and velocity.

c) For optimal results, a prospective dispersal modelling shall be applied leading to a prospective spatial distribution of pollen input in the area. The advantage of this is that only few sample points are necessary to calibrate the gradients of the model at selected sites with the technical pollen samplers delivering a reliable picture of the spatial distribution of pollen exposure over the complete area. Furthermore, scenarios can be modelled for predictions of distinct conditions based on long-term records of the meteorological services, e.g. statistically rare events (10-years, 100-years events), worst case situations, favourable field arrangements, variable buffer zones. Depending on the complexity of the configuration of gm-field(s) and the topography, as a rough estimate about 20 to 50 sample points shall be calculated. This is given by the rationale of five to seven classes of exposure intensity (nonlinear, logarithmic) and four to eight wind sectors. Examples are given in the literature by [8; 17; 26].

d) For simple approaches without dispersal modelling, tests of gradients of exposure by distance from the field(s) at selected directions are possible. At least seven sample points are necessary for testing the gradient in respect to one gm-field. Thus the decline follows a potential function, the sample points shall be equally distributed on a log scale of distance. For more advanced approaches, considering changing wind directions, a y-shaped design using at least 12 sample points is recommended. Both approaches are only possible due to the availability of a broad reference database for this standardized method gained over more than 10 years of field surveys [18]. Without detailed data on weather conditions, e.g. wind direction and velocity, temperature, humidity and thermal stability, and the temporal course of pollen release rates, however, no interferences can be made on the spatial distribution in the area. Examples in the literature are given by [14; 20].

e) For assessment of the spatial distribution of pollen exposure in a region without the help of dispersal modelling, a greater number of sample sites would be necessary. There are various approaches commonly applied for such tasks, the choice of which depends on the sampling objective in detail. A common approach is a sampling grid. A grid is laid over the region; in every grid one sample shall be taken. For example, a region of 10 km × 10 km shall be monitored. A grid of 1 km side length would lead to 100 raster fields; i.e. a number of 100 sample points would be necessary. A grid length of 1 km is very large for pollen dispersal, but a more appropriate grid length of 100 m would already lead to 10 000 sample points. Despite the large number of samples points needed, the results would be poor compared to the approach described under a). Moreover, the linear scale of the grid results in a great number of sites in the outer range with only little

changes in the pollen input and only few sample points at the areas of interest with greater changes at closer distances. In case the location of the gm-fields is known, a nonlinear grid around the gm-fields in the centre, with increasing grid sizes by distance from the field could be applied. However, still large numbers in the order of 100 sample points and more would be required.

6.2.3 Exposure assessment of pollen input for validating and/or calibrating dispersal models

The various pollen species vary in size and shape and differ in their dispersal behaviour in the air. Therefore, a validation and calibration of the dispersion models shall be undertaken in general for any pollen species before application. Secondly, because the release rates of pollen are usually not known in detail, for any application of dispersal modelling a calibration shall be done by field measurements of the pollen input. The number of needed sites depends on the specific task and conditions in the environment; an approximate number in order of 10 to 30 sample points seems appropriate.

6.2.4 General monitoring of pollen exposure at larger scales

— Task: GMO monitoring on larger scales:

This task compares to other fields of environmental monitoring. Due to the disadvantages of static grid designs (see 6.2.2 e)), more advanced approaches might be appropriate, for example by use of dispersal models [26]. In general, the linkage of the sample design for GMO monitoring to the distribution of the pollen sources, e.g. the location of gm-fields, seems obvious. Nevertheless, the changing pattern of crop cultivation shall be taken into account as well as the fact that the cultivation pattern varies from crop to crop and from year to year. This implies a sophisticated sample design, being capable to assess changes in the temporal and spatial distribution of pollen exposure as well as being able to integrate new GMOs. In this context, a prerequisite for any monitoring method is a standardized measurement of exposure guaranteeing comparable data, as described in the present Technical Specification.

6.2.5 Assessment of standardized and acceptor specific pollen deposition

By definition, deposition depends on the condition of the acceptor surface and of the site. Even with the same aerial concentration, a respective variation of deposition rates among different acceptors is observable. For comparable data, a standardized measurement is required as described in the present Technical Specification. For some tasks in GMO monitoring, the knowledge of acceptor specific deposition is desired, e.g. risk assessment of pollen exposure to non-target organisms (for example the deposition of Bt-maize pollen on the leaves of food plants of butterfly larvae). Due to the multiple variable conditions, measurements of specific deposition can be done only punctually and taking into account a great variability. They are not suitable for comparative assessments of pollen input between different sites and different years. Furthermore, sampling the specific deposition needs a lot of time and effort per site compared to the standardized measurement. Therefore any acceptor specific measurements shall always be accompanied by a standardized measurement of pollen exposure to ensure comparable data. Otherwise the results cannot be evaluated properly and compared to findings from other investigations.

6.3 Site conditions

The choice of an appropriate sampling site depends on the intended monitoring objective. For monitoring strategy and sampling design, see 6.2. In this respect, it is of particular importance to distinguish between:

a) standardized surveys (normal case in GMO monitoring), e.g.:

   1) for exposure assessment of sites at local, regional or larger scales in a comparable way;

   2) for validation of forecasts:

The standardized survey is intended to adequately represent the spatial variability of pollen and GMO exposure in a comparable way from site-to-site in the region monitored with respect to the given objective. For GMO monitoring the exposure, as intensity of pollen input of the target GMO, and the setting of the natural-landscape are of particular relevance; this also includes the particular consideration of cultivated areas as a source of pollen release, potential reactants, and protected resources.

In this respect, comparable conditions at the sites are a prerequisite. Unobstructed air flux is important and advantageous when installing the sampler. In addition, the following aspects should be considered:

3) avoidance of close proximity to sources that have no connection with the monitoring objective (i.e. the distance to large pollen sources such as coniferous trees shall be > 100 m);

4) avoidance of sites with wind-tunnelling (or jet stream) effects or sites with shielding effects (buildings, forest edges, hedges; this implies distances more than twice as high as the obstacle);

5) avoidance of sites with untypical topographical characteristics (slopes, dips, particularly exposed ridges);

6) the sampler should not be positioned in the dripping area of trees, houses, or electricity supply lines, or close to other sources of interference (ground excavation, erosion or similar);

b) surveys of pollen dispersal for specific conditions at particular target sites (in individual or special cases), e.g. determination of pollen input for particular reactants or protected resources, individual cases of conflict, special characteristics in vegetation and topography (such as different vegetation types, dells, saddles, ridges, hedges, and buildings), forest clearings and edges, etc.;

In these cases, the monitoring site should represent the particular site conditions.

As far as the monitoring objective allows it, sites protected from vandalism (e.g. fenced-in monitoring stations, private property) might be preferably chosen. Coupling the sampling equipment with apiaries that are designed to be sites for the biological sampling in the GMO pollen monitoring process ([30]) has been proven to be especially advantageous.

6.4 Installing the equipment

— The pole is inserted vertically into the ground until the stabilizing rod is reached (depth of approx. 0,5 m; lower edge of sampler 1,5 m high, upper edge 2 m above ground; see Figure 1), and fixed (using the stabilizing rod); check for stability.

— Mount and orientate the Sigma-2 passive sampler with the PMF onto the floor stand.

— Connect tube between PMF and collection flask.

— Label the collection flask, cover in aluminium foil to protect it from light to avoid growth of algae and fungi, and place it into the sheath.

— Check whether fluid can drain freely from the PMF into the collection flask (1° gradient in the direction of the outlet at the PMF base to the connecting tube; see Figure 1, part 6).

— Prepare site protocol.

6.5 Exposure time

The exposure time depends on the intended monitoring objective.

In general, for GMO monitoring the exposure period shall at least cover the relevant flowering phases of the selected target GMO in the course of the year to be able to assess the total pollen input at the site per season. The passive samplers are optimized with respect to collecting sufficient sample material in as few sampling periods as possible to keep the number of samples per site and season small. In general sampling periods of around four weeks are recommended. Depending on the duration of the blooming phases in the season, one or more successive, individual sampling periods T_e are necessary to cover the total exposure time T per season/year.

The microscopic pollen analysis of the samples shall lead to the total pollen input per season/year and can be performed on the pooled sample for direct results. However, for further tasks in order to get some temporal distinction the analysis might be performed on the individual samples, too. The total input can be calculated by summing up the input. The molecular-biological analysis of GMO by PCR/ELISA should be based on the pooled sample to ensure best performance due to detection limits.

Particular tasks, however, might require shorter or longer exposure periods, resulting in respective changes of pollen counts per sample and the number of samples to be handled.

— Sigma-2 passive sampler:

A sampling period of regularly four weeks is recommended for the Sigma-2 passive sampler. In regard to some particular tasks shorter periods down to one week are possible, depending on the intensity of the expected pollen input for the target GMO. Longer sampling periods give rise to congestion leading to particle agglomeration on the acceptor which makes microscopic identification of pollen species more difficult. On the other hand, a sufficient amount of pollen is required to minimize counting errors. Therefore, a sampling period shorter than one week is not recommended because of insufficient sampling efficiency. Thus, depending on the intensity of pollen input it may be advisable to extend or shorten sampling periods.

— PMF:

A sampling period of around four weeks proved to be of value for the PMF. In order to guarantee satisfactory sample quality, a sampling period of more than six weeks is not recommended. A sampling period shorter than one week is not recommended because of insufficient sample amounts.

6.6 Sampling at site

6.6.1 Sigma-2 passive sampler

The adhesive-coated microscopic slides do not require any special pre-treatment: slides are taken from the dust-tight shipping boxes, labelled and inserted into the clamping system of the Sigma-2 sampler. After exposure the slides are directly put back again to the dust-tight boxes for transport.

For insertion and exchange of adhesive foils or slides, the Sigma-2 passive sampler is opened as shown in Figure 1.

6.6.2 PMF

Under dust-free conditions (laboratory) a set of eight new filtering discs per PMF unit is stacked into the transportation container[5)] in the reverse orientation to that in the PMF (compressed side topmost,

5) Suitable containers: containers with lid, diameter greater than 8 cm and height greater than 10 cm, made of glass (e.g. ¾ l Sturzform J. Weck GmbH, 79656 Wehr) or PP/PE (e.g. PP Becher konisch mit Deckel 1 000 ml, Fa. Rotert GmbH, Osnabrück).

loose side bottommost), and the container is sealed. For the initial insertion of the filtering discs into the PMF at the monitoring site, the conical top is screwed off and the transportation container containing the filtering discs is placed on the top, so that the discs are correctly oriented inside the PMF (compressed side downwards). The container is then removed, and the conical top is attached again. The weight of the 1,5-l PET collection flask is determined (±1 g) and recorded. The collection flask is protected from light by aluminium foil, attached to the connecting tube, and placed into the sheath.

For exchange of samples at site, the conical PMF top is screwed off and filtering discs are transferred into an empty transportation container using a removal instrument (2-pin fork, U-bended steel wire with flattened pins to lift up the filter rods from the bottom of the base). After disconnection from the connecting tube, the collection flask is removed from the sheath and weighed (without closure and aluminium foil). The liquid is visually checked for turbidity, staining, and contaminants. The flask is then re-connected and the conical outlet of the PMF is carefully rinsed with approx. 50 ml to 100 ml of rinsing solution (0,1 % Tickopur[6)]), so that any remaining particles are drained through the tube into the collection flask. It is then disconnected and weighed again, tightly sealed for transportation, and wrapped in aluminium foil for protection against light.

Samples shall be stored light-protected and in the cold (<8°C). Sample preparation shall be carried out within three days after sampling.

6.7 Sample preparation

6.7.1 Preparation of slides for microscopy

The preparation of slides is carried out depending on the analytical tasks.[7)]

For standard qualitative and quantitative visual microscopic pollen analysis in the context of this Technical Specification, the microscopic slides coated with adhesive are prepared by directly applying the embedding medium on the two acceptor fields of 18 mm × 18 mm using a pipette and covering it with a cover slip (preferable size 24 mm × 55 mm). As embedding medium a PVA[8)]-glycerol-solution is recommended with safranine or fuchsine as staining (35 g PVA in 100 ml distilled water over night, adding 50 ml glycerol (85 %) and 10 mg safranine, homogenization, store in small pipette flasks of 30 ml to 50 ml for usage). For removal of air and for constant pressure, a small weight on the cover is applied (e.g. a hexagonal screw nut, size 20) for one hour. Excess embedding medium is removed by carefully using a single edge safety razor blade. Remains on the glass surface of the slides disturbing the microscopic analysis can be removed using cotton swabs and ethanol. The analysis can be done already after about one hour. Using this medium, no extra sealing is necessary, but for drying out and hardening the slides shall be stored horizontally for at least one week. There are alternative embedding media possible, like glycerine-gelatine based ones.

For semi-automatic or automatic pollen analysis using fluorescence microscopy, the same preparation procedure can be used. The water available in the embedding medium enables a controlled swelling of the partially dehydrated pollen, which facilitates the identification of pollen species. Swelling of pollen is prerequisite for automated pollen identification.

Containers are manufactured by the suppliers mentioned above. They are examples of a suitable product. This information is given for the convenience of users of this European Technical Specification and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

6) It is an example of a suitable product. This information is given for the convenience of users of this European Technical Specification and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

7) For further information, see VDI 2119.

8) Polyvinylalcohol: e.g. Mowiol, Gelvatol. It is an example of a suitable product. This information is given for the convenience of users of this European Technical Specification and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

6.7.2 Preparation of PMF samples

Following exposure in the field, pollen samples are extracted from filter units and collection flasks in the laboratory and deposited on a suitable matrix. Pollen particles processed in this fashion are then stored in a suitable form for subsequent proper analyses.

For monitoring tasks dedicated to selected pollen types and GMO (GMO-specific monitoring), specific preparation procedures may be applied optimizing detection limits and results for this GMO. For examples, see Annex A for maize. In the following subclause, a general procedure is described based on glycerol suspension as matrix:

Due to the viscosity of the solution particles are kept in suspension, and untimely or non-uniform sedimentation of particles is avoided, so that representative subsamples can be taken by pipetting for various analyses.

The preparation of PMF pollen samples from the filter pads and the liquid contained in the collection flasks is carried out in the laboratory which makes use of ultrasound and vacuum filtration to sediment the sample as filter cake on membrane filters (Ø 50 mm, 12 µm, sterile). Intermediate storage of membrane-filter (MF) samples is possible at −20°C.

The following procedure is recommended:

— First, the supernatant in the 1,5-l PET collection flask is inserted in the vacuum filtration without agitating it, leaving a rest of approx. 50 ml. If the liquid level is too low, this step is omitted, correspondingly filling with 0,1 % Tickopur[9)] rinsing solution.

— Eight filter pads are each flushed 30 s in beakers (Ø 90 mm) using 0,1 % Tickopur solution in ultrasonic bath. Hereby, the filter pads are squeezed mechanically several times, and the liquid is transferred together with the aerosols continuously into the vacuum filtration via a connecting hose. This procedure shall be repeated. Then, the containers are rinsed.

— Finally, the remaining content of the 1,5-l collection flask is well shaken, treated during 30 s in ultrasonic bath and, after shaking it again, transferred to the vacuum filtration with post-rinsing.

— If the filter gets clogged, it is to be replaced.

The membrane filter samples, as result of the recovery, are documented under the binocular eyepiece with sufficient magnification.

Pollen samples (from filter cake) are then transferred into 4 ml of approx. 50 % glycerol suspension (density approx. 1,1 g/cm^3) contained in a sealable centrifuge tube suitable for PCR. The pollen suspension (PS) is then mixed on a shaker, and the following subsamples for various analyses are pipetted:

— a subsample for microscopic pollen analysis (UM) for qualitative analysis of the pollen diversity and determination of pollen total counts for the number of specific target pollen (see 7.3): 1 × 300 µl in 0,5-ml tube (Safe-Lock tubes, e.g. from Eppendorf[10)]);

— two subsamples for PCR analyses: 1 000 µl each (UP_{a-b}), in 2-ml tubes, respectively (safe lock);

9) It is an example of a suitable product. This information is given for the convenience of users of this European Technical Specification and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

10) It is an example of a suitable product. This information is given for the convenience of users of this European Technical Specification and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

— residual suspension: retain sample (UR).

Corresponding to the result of the microscopic pollen analysis (UM), the pollen samples shall be separated from air-dust particles for improving the analytics via additional preparation steps, and certain pollen fractions shall be concentrated with the target pollen types, whereby another quantitative microscopic analysis of the target pollen types shall be carried out for validating the result. See Annex A for maize pollen as example.

The conditions for the basic steps of the treatment (recovery of the pollen samples in ultrasonic bath with subsequent vacuum filtration and transfer into a glycerol suspension) shall be kept as follows:

— During the entire procedure, contamination shall be avoided and the completeness of the samples shall be guaranteed.

— Recovery rate of >98 % for pollen aerosol parts 10 µm to 150 µm. It should be determined via application of reference pollen suspensions of the target pollen type(s) with known dose.

— Integrity of the pollen (>98 %). It should be determined by means of a microscopic check of the pollen material in the filter cake and in the quantitative counting of the final sample (UM).

— At regular intervals, blank samples, at least before the first and after the last sample of a series, shall be carried out.

The pollen suspension PS and subsamples can be stored at −20 °C.

7 Microscopic pollen analysis

7.1 General

For identification of pollen types and quantification of pollen input microscopic pollen analysis is applied.

Pollen reference slides prepared for any relevant pollen type are the most important tool for identification. Further literature on pollen species with microscopic pictures is essential, commonly named as “pollen atlas” [9; 10; 22; 27]. Identification keys for acetolyzed pollen – which are common for fossil pollen in Palynology – are unsuitable, since their appearance in microscopic mounts differs significantly; however they are useful showing exine structural elements in more detail. Furthermore, pollen atlases are available online such as [23]. Taxonomic naming of plant species should be carried out according to [13].

7.2 Sigma-2 passive sampler

7.2.1 Microscopic imaging methods

Transmitted-light bright-field microscopy is used for pollen analysis. Various pollen species are differentiated on the basis of their characteristic morphologically and structurally distinguishing features such as size, shape, texture and structural elements of the exine like pores, plasma structure, etc.

Apart from pollen, dust samples may contain further biological particles, such as:

— other intact plant entities (spores, algae),

— plant fragments (trichomes, leaf and seed hairs),

— plant residues (wood fibres (cellulose), leaf and cell fragments, plant-tissue residues),

— insect fragments (wing fragments, leg fragments, claws, hair (chitin)) and/or

— animal and human remnants (hair (protein), epithelial layers).

7.2.2 Qualitative analysis of the pollen diversity

The evaluation of a qualitative analysis of pollen types is carried out in table form, and can be displayed as a cumulative frequency distribution spanning the sample collective and the diversity of pollen types.

7.2.3 Quantitative analysis of the pollen

Microscopic counting of samples collected with the Sigma-2 passive sampler yields the value $N_{i,\mathrm{Sigma}}$ (T_e) for the particle deposition at the site. The measured value is defined as: pollen count N of pollen species i on counting area A for exposure period e and time T_e (time in d).

The chosen counting area A should give a representative counting result for the pollen species in the sample. Choice of the counting area depends on the acceptable counting error, the detection limit of the method and density of pollen presence (dynamic counting). A minimum counting area of 200 mm^2 shall be analysed, in order to gain a minimum total pollen count of 1 000 pollen grains. If this number is not reached, the counting area is to be extended accordingly. The counting area (A_i) can be extended with regard to particular target pollen species that are present only in minor amounts (e.g. maize pollen), without the need to count all pollen species. Thus, the detection limit is improved, and both effort and sampling error, which gets very large for counts $n < 3$ (Poisson distribution), are reduced. Quantification of undetectable pollen species is given as < 1 and not as zero value, the related counting area shall accounted for, so that detection limits can be specified.

7.3 PMF

7.3.1 Microscopic analysis

For qualitative and quantitative microscopic pollen analysis the subsample of the pollen suspension (UM) is used (as described in 6.7.2). The following procedure was developed by the LAVES Institute for Apiculture Celle based on the preparation steps for pollen analysis in honey as described in DIN 10760 [2].

For microscopic imaging the same method is used as described above in 7.2.1.

7.3.2 Qualitative analysis of the pollen (diversity)

The evaluation of a qualitative analysis of pollen types is carried out in table form, and can be displayed as a cumulative frequency distribution spanning the sample collective and the diversity of pollen types.

7.3.3 Quantitative microscopic analysis of pollen

Microscopic pollen analysis is used for determination of pollen counts per pollen types and sample. The presentation in this subclause refers to the data analysis of a single sampling period *e*. However, for the purpose of simplifying notation, explicitly mentioning the respective sampling period is – as far as possible – avoided.

The total pollen count as well as pollen counts of particular species that are relevant for GMO monitoring (e.g. rape, maize, etc.) and, depending on the season, of particular key species (coniferous trees, oak, grass species), are determined. A relative detection limit of 0,1 % should be adhered to. Therefore, counting of a minimum of 1 000 pollen grains is required. Application of the dynamic counting method is recommended:

Using a micropipette, 10 µl are taken from the subsample of the glycerol pollen suspension for microscopy (UM) and transferred onto a microscopic slide (shake UM sample before pipetting), covered with a cover slip (22 mm × 22 mm), and the total pollen count is estimated. If necessary, the glycerol

pollen suspension (UM) is adjusted after this, i.e. by adding glycerol (GZ) so that a volume of 10 µl to 50 µl taken from it results in approximately 1 000 pollen grains (dilution factor f_z) in the counted sample (ZU). Several subsamples (M_k, k = 1; 2; …; $K \geq 2$) with corresponding volumes of 10 µl to 50 µl are subsequently taken and used for counting.

Using a 400-times magnification, counting of the respective target pollen species and determination of total pollen count is carried out, so that pollen species of interest per mount are counted completely. Mounts are successively counted until a minimum of 1 000 pollen grains in total are identified. For this, at least two mounts are analysed – a third or further mounts should be analysed where necessary. The pollen count of the counted volumes is then extrapolated to the total sample, in which the respective total pollen count relating to the PMF sample is specified for individual pollen species and total pollen.

The absolute detection limit for the pollen counting is determined by counted subsamples that contain one pollen grain. Samples containing less pollen of a particular target species than the respective absolute detection limit, should be specified as < 1 and not as zero value; the related counting area shall be accounted for, so that specific detection limits can be specified.

First, the quantity to be measured is determined as pollen counts that relate to the total volume counted, i.e. by adding pollen counts and volumes of mounts counted (k):

$$N_{i,z} = \sum_{k=1}^{z} N_{i,k} \text{ in } n \text{ pollen} \tag{1}$$

$$V_z = \sum_{k=1}^{z} V_k \tag{2}$$

where

$N_{i,z}$ is the total pollen count of species i in z microscopic mounts counted (recommendation: $\sum_i N_{i,z} \geq 2000$) (counted value);

$N_{i,k}$ is the pollen count of species i in microscopic mount M_k (k = 1, 2, ..., K);

V_z is the total volume of z microscopic mounts counted; in µl;

V_k is the volume of microscopic mount k (k = 1, 2, ..., K);

i is the index of pollen species;

k is the index of microscopic mount (k = 1, 2, ..., K);

K is the total number of microscopic mounts (recommendation: $K \geq 2$);

z is the number of microscopic mounts actually counted ($z \leq K$; recommendation: $z \geq 2$).

Pollen concentrations $C_{i,\mathrm{PS}}$ for individual pollen species and total pollen in the glycerol-suspension sample (PS) from the PMF are calculated according to the following steps and taking the dilution factor f_z into account:

$$f_z = \frac{V_{\mathrm{ZM}}}{V_{\mathrm{UM}}} = \frac{V_{\mathrm{UM}} + V_{\mathrm{GZ}}}{V_{\mathrm{UM}}} \tag{3}$$

$$N_{i,\mathrm{UM}} = f_z \cdot N_{i,z} \text{ in } n \text{ pollen} \tag{4}$$

$$C_{i,\mathrm{PS}} = C_{i,\mathrm{UM}} = \frac{N_{i,\mathrm{UM}}}{V_{\mathrm{UM}}} \text{ in } n \text{ pollen/µl} \quad (5)$$

where

f_z is the dilution factor of counted suspension;

V_{UM} is the volume of microscopy subsample UM (taken from PS);

V_{ZM} is the volume of suspension adjusted by dilution of UM;

V_{GZ} is the volume of glycerol added;

$N_{i,z}$ is the total pollen count of species *i* in *z* microscopic mounts counted (recommendation: $\sum_i N_{i,z} \geq 2000$) (counted value), taken from Formula (1);

$N_{i,\mathrm{UM}}$ is the pollen count of species *i* in microscopy subsample UM;

$C_{i,\mathrm{PS}}$ is the pollen concentration of species *i* in pollen suspension sample (required);

$C_{i,\mathrm{UM}}$ is the pollen concentration of species *i* in microscopy subsample UM.

The absolute pollen count of species *i* in the PMF sample is then obtained from pollen concentration $C_{i,\mathrm{PS}}$ as follows:

$$N_{i,\mathrm{PMF}}(T_e) = N_{i,\mathrm{PS}} = C_{i,\mathrm{PS}} \cdot V_{\mathrm{PS}} \text{ in } n \text{ pollen} \quad (6)$$

where

$N_{i,\mathrm{PMF}}\ (T_e)$ is the pollen count of species *i* in PMF sample (required);

$N_{i,\mathrm{PS}}$ is the pollen count of species *i* in pollen suspension sample PS;

$C_{i,\mathrm{PS}}$ is the pollen concentration of species *i* in PS, taken from Formula (5);

V_{PS} is the volume of pollen suspension sample PS.

8 Molecular-biological analyses of GMO

Pollen of GMO contain specific DNA (transgene DNA) and/or specific proteins/toxins. For detection of pollen deriving from GMO, molecular-biological analytical methods are used. For analysis of DNA in pollen samples, quantitative PCR (Polymerase Chain Reaction) is applied, for analysis of proteins/toxins ELISA (Enzyme-linked immunosorbent Assay).

For monitoring tasks dedicated to selected pollen types and GMO (GMO-specific monitoring), specific analytical procedures should be applied optimizing detection limits and results. For optimal results, molecular-biological analysis of pollen using PCR and/or ELISA requires pollen-type specific preparation and event-specific analysis. For examples see Annex A for maize. It shall be mentioned that the molecular-biological analysis of pollen goes far beyond routine-analysis. A successful performance requires to establish the method specifically.

In the following, a general, non-pollen type-specific, procedure aiming for DNA-analysis using PCR is described based on glycerol suspension as matrix:

PMF subsamples ($UP_{a\text{-}b}$) are taken for the analysis of a possible GMO input. Using PMF, pollen samples can be obtained in sufficient quantity and quality for molecular biological tests such as PCR.

By means of the quantitative microscopic pollen analysis of PMF samples obtained in several years of field tests (2001 to 2005, several hundred samples), it could be proven empirically that the pollen from PMF samples is intact. If carried out in a proper form, pollen germination, burst pollen, mould, and algae formation are relevant for the result neither during collection nor during treatment.

The basic preparation of the recovery of the pollen samples is described in 6.7. Depending on the analysis method – e.g. for samples with high content of other aerosols or low proportions of target pollen – it might be required to carry out certain additional preparations such as cleaning and concentration of the target pollen before extracting the DNA and analysing it. For example see Annex A.

The further steps of DNA extraction and analysis using PCR is the same as described in Annex A.

Other molecular analysis methods such as ELISA targeting on proteins and toxins specific for the GMO are also possible, but not described here.

9 Determination of the target parameters for GMO monitoring and representation of the results

9.1 General

In view of the evaluation in GMO monitoring, the courses of pollen rates (Sigma-2 passive sampler: deposition; PMF: flux) throughout the individual sampling periods as well as accumulated total deposition (Sigma-2 passive sampler) and accumulated flux (PMF) throughout the flowering phases per year should be determined and presented separately for each pollen species. For comparable presentation of the results, time (x-axis) should be displayed in linear and the value axis (y-axis) in logarithmic scale.

9.2 Sigma-2 passive sampler

9.2.1 Determination of pollen deposition per sampling period

The determination of pollen deposition rates per sampling period is aimed for total pollen and for the pollen species *i* that are relevant for GMO monitoring.

From the measured value $N_{i,\mathrm{Sigma}}\ (T_e)$ (pollen count per selected area of the specimen; see 7.2.3) the pollen deposition per m² $D_{i,\mathrm{Sigma}}\ (T_e)$ over the exposure time T_e of sampling period *e* can be calculated by referring the pollen count to the standard reference area of 1 m².

$$D_{i,\mathrm{Sigma}}(T_e) = \frac{N_{i,\mathrm{Sigma}}(T_e)\cdot 10^6}{A} \text{ in } \frac{\text{pollen}}{\mathrm{m}^2 \cdot T_e} \tag{7}$$

where

$D_{i,\mathrm{Sigma}}\ (T_e)$ is the pollen deposition of species *i* per m² during sampling period *e;*

$N_{i,\mathrm{Sigma}}\ (T_e)$ is the pollen count of species *i* on counting area of the Sigma-2 passive sampler in sampling period *e;*

A is the counting area of Sigma-2 passive sampler in mm².

9.2.2 Determination of the daily mean pollen deposition rate per sampling period

The daily mean pollen deposition rate $D_{i,\mathrm{Sigma}}$ (d) of sampling period *e* is calculated according to:

$$D_{i,\mathrm{Sigma}}\ (\mathrm{d}) = \frac{D_{i,\mathrm{Sigma}}\left(T_e\right)}{T_e}\ \mathrm{in}\ \frac{n\ \mathrm{pollen}}{\mathrm{m}^2 \cdot \mathrm{d}} \tag{8}$$

where

$D_{i,\mathrm{Sigma}}$ (d) is the pollen deposition of species *i* per m² during one day of sampling period *e;*

$D_{i,\mathrm{Sigma}}$ (Te) is the pollen deposition of species *i* per m² during sampling period *e;*

T_e is the exposure time of individual sampling period *e*, in days;

d is the sampling unit "day".

9.2.3 Determination of yearly pollen deposition

The pollen deposition of species *i* summed-up over the year $D_{i,\mathrm{Sigma}}$ (a) is calculated for the purpose of GMO monitoring. If the sampling period is shorter than the blooming phase, this shall be specified.

$$D_{i,\mathrm{Sigma}}\ (\mathrm{a}) = \sum_{e=1}^{E} D_{i,\mathrm{Sigma}}(T_e)\ \mathrm{in}\ \frac{n\ \mathrm{pollen}}{\mathrm{m}^2 \cdot \mathrm{a}} \tag{9}$$

where

$D_{i,\mathrm{Sigma}}$ (a) is the pollen deposition of species *i* per m² and year;

$D_{i,\mathrm{Sigma}}$ (Te) is the pollen deposition of species *i* in sampling period *e;*

a is the sampling unit "year";

e is the index of sampling period;

E is the number of sampling periods;

T_e is the sampling time of period *e*, in days.

9.2.4 References to pollen dispersal models

As shown in 10.4 to 10.6, pollen deposition results can be related to results of pollen dispersal modelling. This is of relevance for transferability of results, assessment, and for the determination of numbers of cases required in GMO monitoring.

9.3 PMF

9.3.1 Pollen count per sample $N_{i,\mathrm{PMF}}$

Pollen count $N_{i,\mathrm{PMF}}$ (T_e) gives the total number of pollen grains per sample for individual pollen species or total pollen, respectively, that have been collected in the PMF during the sampling period T_e. It is identical to the pollen count $N_{i,\mathrm{PS}}$ in the pollen suspension as given in 7.3.

The pollen count per sample $N_{i,\mathrm{PMF}}$ (T_e) is a characteristic parameter with respect to the absolute detection limit of PCR analyses.

9.3.2 Relative frequency of pollen species *i*

The relative frequency *H* of pollen species *i* in percent of the total pollen input is calculated using the following formula, in which the input number refers to the corresponding period (d, T_e, a):

$$H_{i,\mathrm{PMF}} = \frac{N_{i,\mathrm{PMF}} \cdot 100}{N_{\mathrm{ges,PMF}}} \tag{10}$$

where

$H_{i,\mathrm{PMF}}$ is the relative frequency of pollen species *i* in PMF sample, in %;

$N_{i,\mathrm{PMF}}$ is the pollen count of species *i* in PMF sample;

$N_{\mathrm{ges,PMF}}$ is the total pollen count in PMF sample.

The relative frequency is a characteristic parameter with respect to the relative detection limit of PCR analyses.

9.3.3 Determination of pollen flux per sampling period

The PMF filters pollen from the air flux and therefore describes a horizontal flux.[11)] The reference area is oriented vertically to the wind direction. The pollen flux in the sampling period *e* provides the number of pollen grains transported to the sampling site by the wind as a standardized unit per m² of the reference area. Pollen flux measured with the PMF $F_{i,\mathrm{PMF}}$ (T_e) for species *i* and for total pollen, accordingly, is calculated according to the following formula:

$$F_{i,\mathrm{PMF}}(T_e) = \frac{N_{i,\mathrm{PMF}} \cdot 10^6}{8 \cdot 10^3} \text{ in} \frac{n \text{ pollen}}{\mathrm{m}^2 \cdot T_e} \tag{11}$$

where

$F_{i,\mathrm{PMF}}$ (T_e) is the pollen flux of species *i* per m² in sampling period *e*;

T_e is the sampling time of sampling period *e*, in days, standard reference area: 1 m² = 10^6 mm² projected collection cross-sectional area of PMF sampler = 8 × 10^3 mm².

9.3.4 Determination of the daily mean pollen flux rate per sampling period

The **daily** mean pollen flux rate $F_{i,\mathrm{PMF}}$ (d) of sampling period *e* is calculated according to:

$$F_{i,\mathrm{PMF}}(\mathrm{d}) = \frac{F_{i,\mathrm{PMF}} \cdot (T_e)}{T_e} \text{ in } \frac{n \text{ pollen}}{\mathrm{m}^2 \cdot \mathrm{d}} \tag{12}$$

where

$F_{i,\mathrm{PMF}}$ (d) is the **daily** mean pollen flux rate of species *i* during sampling period *e;*

T_e is the exposure time of individual sampling period *e*, in days;

d is the sampling unit “day”.

11) This includes all inputs that are directed by air flux into the vertical acceptor area.

9.3.5 Determination of the yearly pollen flux

The yearly pollen flux is an important target parameter of GMO monitoring for assessing the dispersal of plant pollen with a view to the documentation of GMO input and spread. Pollen flux transported by wind over the year $F_{i,\mathrm{PMF}}$ (a) is the pollen flux summed-up over the sampling periods. If the sampling period is shorter than the blooming phase, this shall be specified.

$$F_{i,\mathrm{PMF}}(\mathrm{a}) = \sum_{e=1}^{E} F_{i,\mathrm{PMF}} \cdot (T_e) \text{ in } \frac{n \text{ pollen}}{\mathrm{m}^2 \cdot \mathrm{a}} \tag{13}$$

where

$F_{i,\mathrm{PMF}}$ (a) is the pollen flux of species *i* per year;

$F_{i,\mathrm{PMF}}$ (T_e) is the pollen flux of species *i* in sampling period *e*

a is the sampling unit “year”;

e is the index of sampling period;

E is the number of sampling periods;

T_e is the sampling time of period *e*, in days.

9.3.6 Assessment of results from molecular-biological analyses

The molecular-biological analysis of the samples is carried out according to Clause 7.

When evaluating the results, the specific limits of detection and probabilities of error shall be taken into account for the detection of transgenes and/or toxins/proteins in pollen DNA. This applies in particular to the probability of incorrect negative results, which exist with lower GMO proportions when approaching the limit of detection. Therefore, the individual results of – at least two – repeated measurements shall be listed completely and traceably (number of positive and negative detections).

For the task of GMO monitoring relevant to this Technical Specification, the results of the molecular-biological analysis shall be reported specifically for any target pollen type together with the number of the respective pollen counts per sample and the pollen counts per single aliquot of the analysis. In case of DNA analysis (PCR) the results shall be given as percent gm-pollen to total pollen of the particular pollen type. In case of gm-specific toxin analysis using ELISA, the results shall be reported as µg toxin content per g of respective pollen type.

For GMO monitoring purposes false-negative results are a critical issue. The assessment shall be undertaken to exclude any GMO input at the site as testing hypothesis. The results shall be presented in such a way that the validity can be seen and conflicting results are documented in a reproducible way. The assessment on GMO shall lead at least to three classes:

— “GMO input detected [2+]” in case of consistent positive measurements; in the example here: two.

— “GMO input cannot be excluded [2-, 1+]” in case of conflicting results; for example: two negative and one positive measurements.

— “GMO input not detected [2-]” in case of consistent negative measurements.

9.3.7 References to pollen dispersal models

Results with regard to pollen flux and pollen flux rate obtained from pollen counting using the PMF can be related to results from pollen dispersal modelling. This is of relevance for transferability of results, assessment, and for the determination of numbers of cases required in GMO monitoring. For more details and examples see VDI 4330 Part 3 [31] and [26].

10 Performance characteristics of the methods

10.1 General

The performance characteristics of the sampling method is described in detail in VDI 4330 Part 3 [31]. It includes the validation of the method in terms of accuracy, precision and uncertainty according to ISO 5725-1 [12)] [1] with detection limits, sensitivity and repeatability.

10.2 Validation

The validation was carried out based on data obtained in field experiments according to DIN 32645 [3]. The following test approaches were carried out:

a) Gradients check: The core of the validation of the sampling method was the comparison of pollen flux and deposition, which were measured in a field experiment using PMF and Sigma-2 passive sampler, with the values for flux, deposition and concentration, which were to be expected at the sampler locations based on a dispersal model. The situation and size of the emission areas in the experiment were known. The concentration, deposition and flux expected at the sampler locations were determined by means of a dispersal calculation based on a Gaussian model. The weather conditions required for it were measured at the same time with the sampling of pollen during the experiments in the years 2001 and 2002. The monitoring sites were selected so that they would cover the variation of pollen concentration, deposition and flux starting with the maximum possible values in the areas close to the fields to the reference areas when assuming average weather conditions. Hereby, different wind directions, cultivation densities and regional conditions were taken into account apart from the different distances to the sources. Details on the method and results have been published in [17], the most important results are mentioned in the following sections.

b) Parallel measurements: Measurements in duplicate with each two samplers at several locations were carried out in order to be able to estimate the spreading of the sampler behaviour.

c) Comparative measurements using standard methods: Comparative measurements using the Hirst pollen trap [28] (Burkard[13)]) as standard device of the pollen information services (volumetric active sampler) were carried out so that relations to the absolute pollen concentration of the conventional method could be checked and made possible.

Several conclusions with regard to the validation could be obtained from the above-mentioned measurements and calculations:

— the confirmation that the pollen sampling by means of PMF and Sigma-2 passive sampler gives an image of the immission to be expected;

— furthermore, parameters for sensitivity, detection limits and reproducibility.

12) This document is currently impacted by the corrigendum ISO 5725-1:1994/Cor 1:1998.

13) It is an example of a suitable product. This information is given for the convenience of users of this European Technical Specification and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

From the gradient check, the relation between the expected and measured values of the pollen dispersal was quantified only up to a proportionality factor because the absolute source strength is not known. For a validation in narrower sense, however, only the correlation between expected and measured ambient input is important, which does not depend on this factor and which has been significant and high. Sensitivity, limit of detection and reproducibility resulted from the increase of the mathematical relation between expected and measured ambient input, the hereby estimated background level and the deviation of observed values around the curve describing the relation. The parallel measurements delivered values for the coefficient of variation of the measured values and thus complete the results on the reproducibility.

The comparative measurements with the active device deliver relations to the absolute concentration according to a conventional method and thus allow the comparability of the results with other methods. The determined parameters described in the following subclauses served finally for estimating numbers of cases for future samplings.

10.3 Distribution of measured values

The distribution of pollen count data obtained from measurements using the Sigma-2 passive sampler and the PMF approximately follows a logarithmic Gaussian normal distribution, i.e. it can generally be regarded as a superposition of several logarithmic normal distributions, similar to pollen count data of air-dust samples [11]. Figure 3 and Figure 4 show on a logarithmic scale the measured values, non-parametric density estimations (kernel density estimator with Gaussian kernel) [24], and normal distributions adjusted to the left flank of the density estimation. Therefore, a corresponding logarithmic data transformation is required for the analysis of pollen count values. The shoulder visible on each left flank is characteristic for the concentration of sites close to the fields.

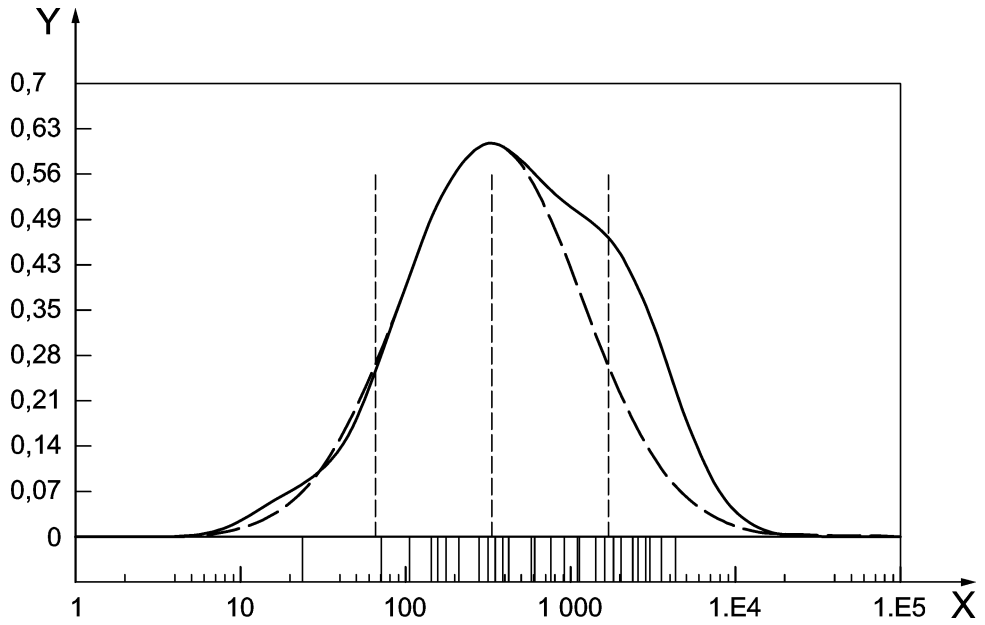
10.4 Methodical approach and determination of basic parameters

The following presentation is confined to the main features of the procedure; justifications and technical details are described in [17].

The correlation between the pollen count observed and expected pollen flux or concentration at the monitoring site was modelled in a “hockey-stick” approach [15; 29]. In doing so, a predefined background input is assumed at sites far from a source, therefore giving a constant pollen count on the samplers at these sites except for accidental variations. In contrast, in approaching a source, the expected pollen count on the sampler starts to increase at a particular point.

Absolute detection limits of technical samplers are defined by the condition that a sample to be counted shall at least contain one pollen grain. The usual procedure for the determination of detection limits (calibration curve method) was modified for this purpose.

Furthermore it shall be considered that a subsample was usually used for counting: 2 % to 5 % (PMF) or 250 mm^2 = 6 % (Sigma-2 passive sampler).

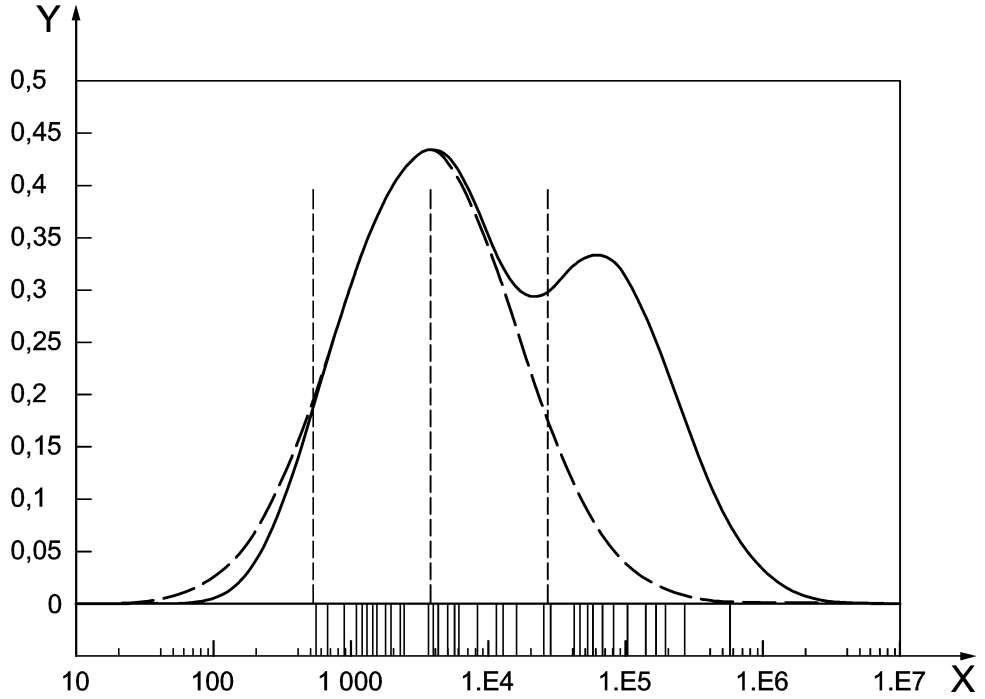


Key

Y density

X Rapa pollen count on Sigma-2 adhesive foil (4 225 mm^2)

Figure 3 — Distribution of measured rape pollen counts using the Sigma-2 passive sampler and density estimation with log-normal base distribution [17]



Key

Y density

X Rapa pollen count on PMF (0,008 m^2)

Figure 4 — Distribution of measured rape pollen counts using the PMF and density estimation with log-normal base distribution [17]

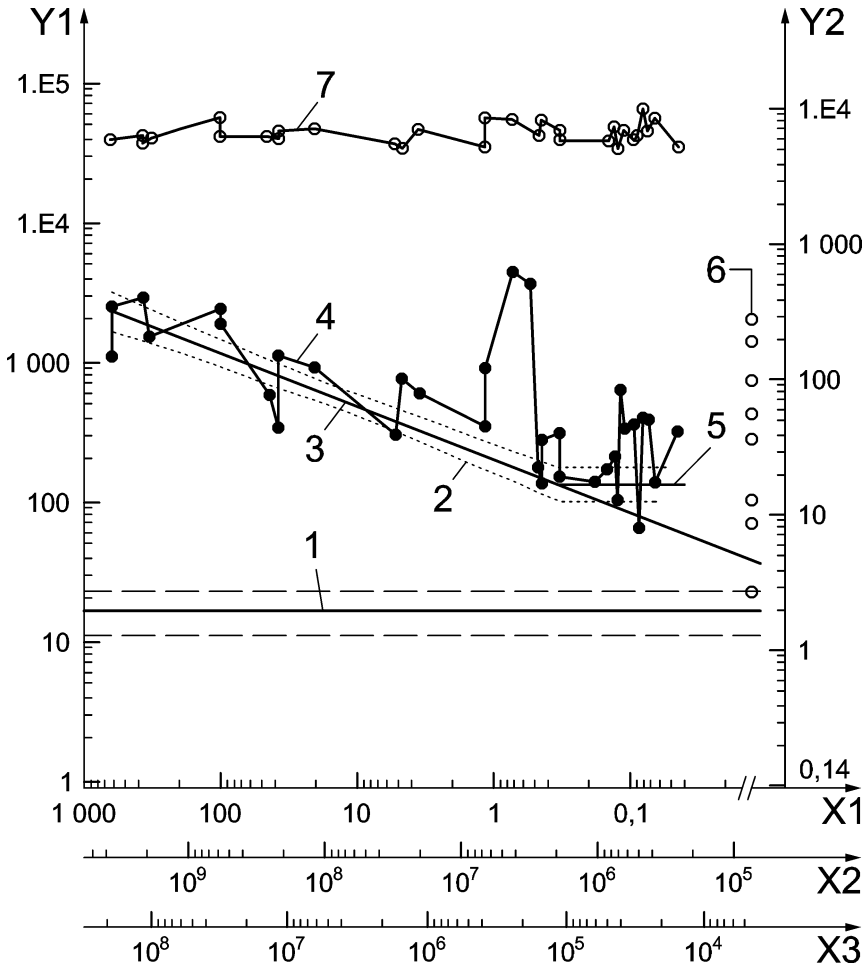
10.5 Sigma-2 passive sampler

10.5.1 Sensitivity, detection limit and reproducibility

The following subclause describes the performance of the sampler and the measurement uncertainties.

Validating the Sigma-2 passive sampler was carried out according to the method that uses the comparison with gradients derived from the dispersal model as described above. Results from experiments carried out during the rape blooming period at 49 sites are displayed in Figure 5.

As expected, total pollen counts measured fluctuate around a constant level independent of the relative position to the GMO rape fields. Rape pollen counts, however, decrease from left to right until a regional baseline level is reached, around which values remain stable. Sensitivity of the Sigma-2 passive sampler results from regression analysis that proves a statistically significant correlation ($P < 0{,}001$) between rape pollen deposition on adhesive foils of the Sigma-2 passive sampler and the gradient resulting from the dispersal model (decreasing line). Reproducibility is represented by the confidence band (between dotted lines). Since rape pollen counts measured with the Sigma-2 passive sampler reflect the total rape pollen input from the experimental and other regional rape fields, individual sites in close proximity to other rape fields show higher values accordingly. In more distant areas with less additional input from GMO fields, the curve of rape pollen counts sways like a “hockey stick” onto a level defining the baseline rape pollen input that corresponds to the density of the regional rape cultivation (horizontal line with confidence band). Reference sites are located in different regions and include sites that are far away from any kind of rape cultivation (e.g. Bavarian Forest). Rape pollen counts at reference sites show a comparable range of measurement with an extension in the lower dose range – in which the detection limit was reached by a Sigma-2 passive sampler using the former counting area standard of 250 mm^2 – and are indicated by the horizontal line with confidence band.



Key

1 detection limit

2 reproducibility (confidence band)

3 sensitivity (slope of regression line)

4 rape pollen

5 regional baseline level

6 rape pollen reference areas

7 total pollen

Y1 pollen deposition on Sigma-2 adhesive foil over exposure time T (n pollen/(4 225 mm^2 x T))

Y2 mean pollen deposition rate per m^2 and day in the exposure period T (n pollen/(m^2 x d)

X1 mean pollen concentration per m^3 air during exposure time T acc. to dispersion model (n pollen/m^3)

X2 pollen flux over exposure time T acc. to dispersion model (n pollen/(m^2 x T))

X3 pollen deposition over exposure time T acc. to dispersion model (n pollen/(m^2 x T))

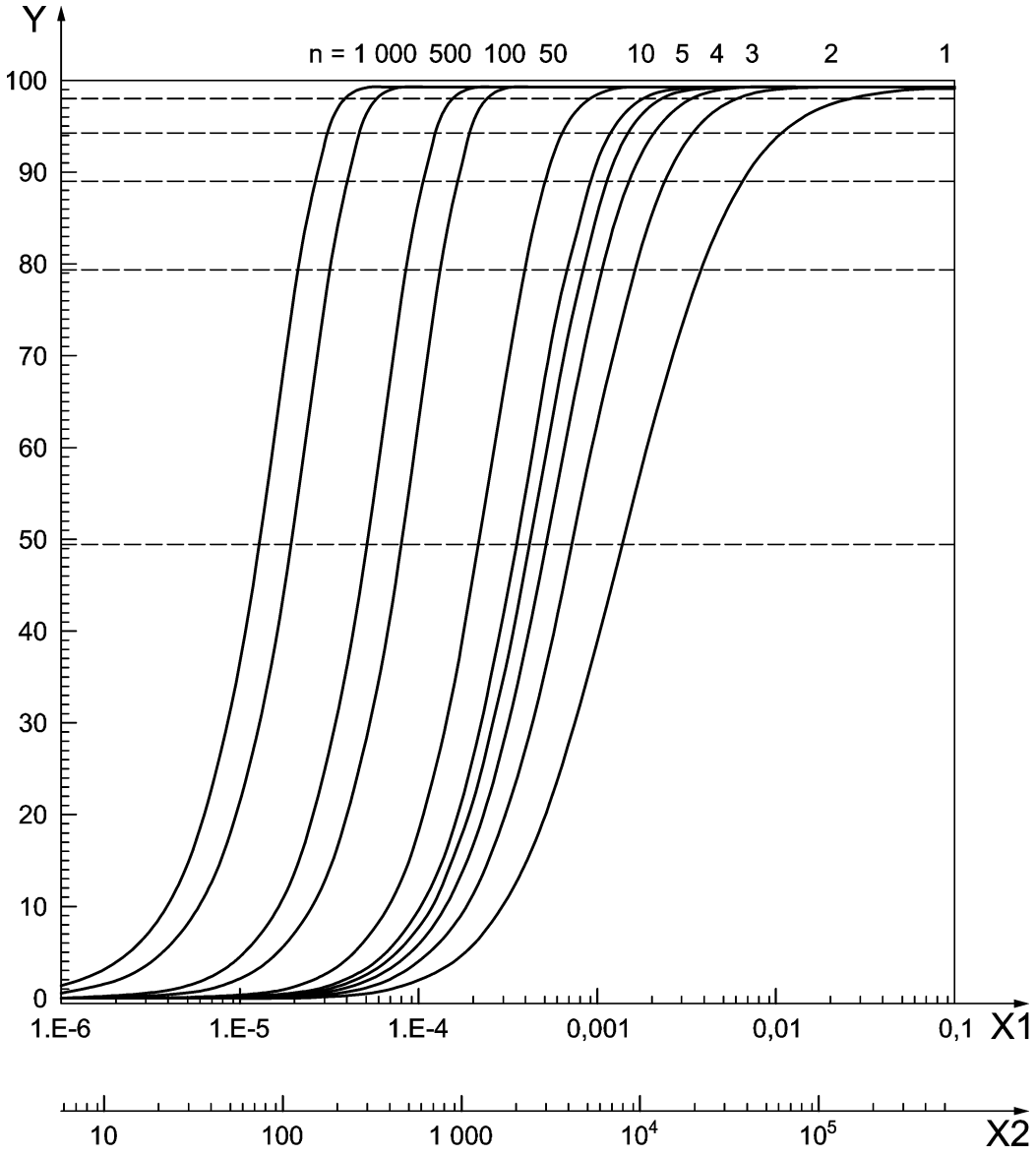
The lower axes indicate predicted values from the dispersal modelling for the mean rape pollen concentration over the exposure time T = 34 d (X1), the respective pollen flux (X2), and deposition (X3) (at v_d = 0,01 m/s). Vertical axes designate the pollen deposition as measured with the Sigma-2 passive sampler: the right axis (Y2) indicates the mean pollen deposition rate per m^2 and day, the left (Y1) the pollen deposition on the Sigma-2 adhesive foil during the exposure time of 34 d in this case.

Figure 5 — Sensitivity, detection limit and reproducibility for the Sigma-2 passive sampler [17]

10.5.2 Detection confidence level and required numbers of cases

Figure 6 shows the confidence level of the detection method with regard to pollen count for various numbers of cases. The vertical axis represents the detection confidence level (defined as the probability for ability to detect the value given on the horizontal axis). Pollen deposition (in rape pollen m^2/T) is displayed on the first horizontal axis; the second scale refers to the corresponding mean rape pollen concentration as determined in dispersal model for the boundary conditions of the rape experiment of 2001 (blooming phase: 34 d; mean wind velocity: 1,93 m/s, $Q = 10^9$ pollen/(ha × h)).

For the Sigma-2 passive sampler the 50 % detection confidence level is given at a pollen deposition of approximately 7×10^3 pollen grains/m^2/T, corresponding to a mean pollen concentration of approximately 0,0015 pollen grains/m^3 over 34 days, or approximately 0,04 pollen grains/m^3 over seven days. As illustrated by the curve, the detection confidence level increases with increasing pollen deposition. The 95 % detection reliability for the Sigma-2 passive sampler is reached at pollen deposition of approximately 5×10^4 pollen corresponding to an average concentration of approx. 0,01 pollen grains/m^3 over 34 days or 0,2 pollen grains/m^3 over seven days. As shown, detection limit and validity are further improved by increasing the number of cases.



Key

Y detection confidence level (in %)

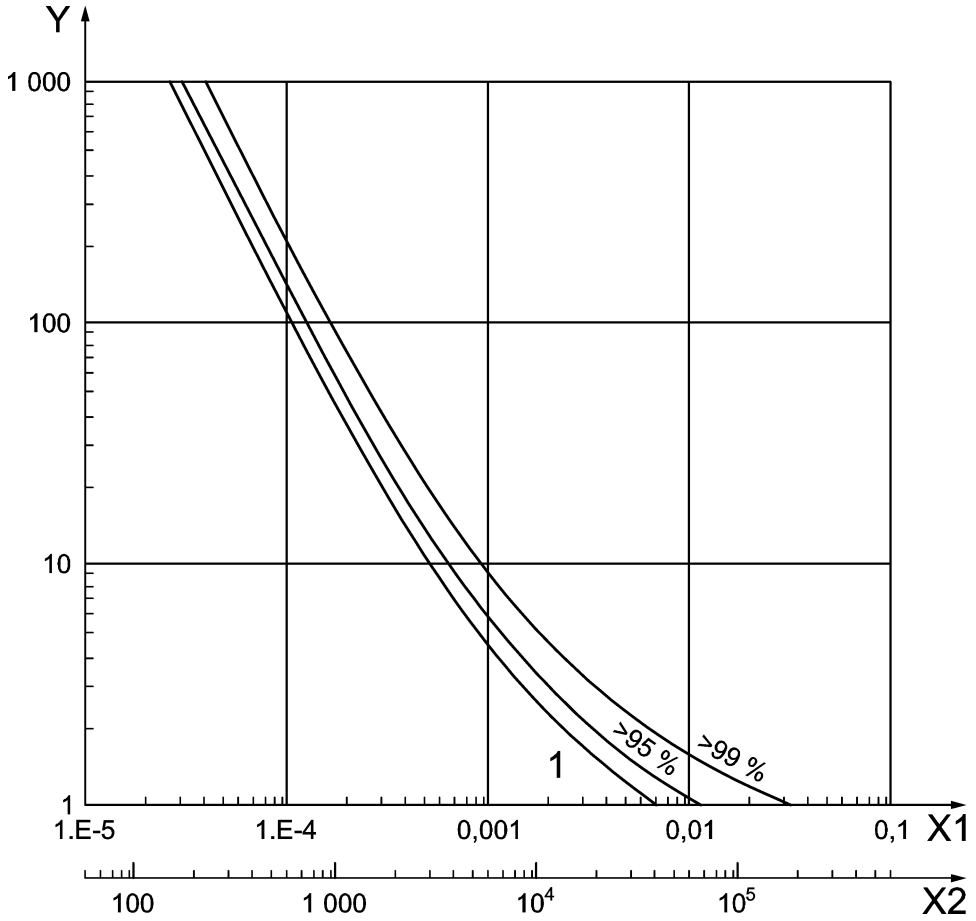
X1 pollen concentration to be detected (n pollen/m^3) with v = 1,93 m/s

X2 pollen deposition to be detected (n pollen/m^2/T)

Figure 6 — Detection confidence level of the passive sampler Sigma-2 for rape pollen against pollen concentration and pollen deposition, respectively, and various numbers of cases required [17]

To keep the detection limit and validity to a distinct level in a monitoring for all sites, this may be achieved by increasing the counting area (see 7.2.3) and the number of samplers per site according to the intensity of pollen flow expected at the sites. Figure 7 shows the number of cases required for GMO monitoring for chosen detection confidence levels. An acceptor surface of 250 mm^2 being analysed microscopically has been taken here as one case unit. On the horizontal x-axis the detection limit is stated in concentration and deposition units. The vertical y-axis denotes the number of cases that are required to keep a distinct detection probability indicated by the curves.

NOTE Usually, a microscopic mount has a potential acceptor surface for good microscopic analysis of up to 1 000 mm² (this takes space for labelling and edges into account). The adapter of the Sigma-2 is designed for holding up to two slides. This gives a maximum area of 2 000 mm² per one Sigma-2 sampler fully equipped with 2 slides.



Key

1 detection confidence level > 90 %

Y number of samplers

X1 average pollen concentration to be detected (pollen/m³)

X2 pollen deposition to be detected (pollen/m²/*T*)

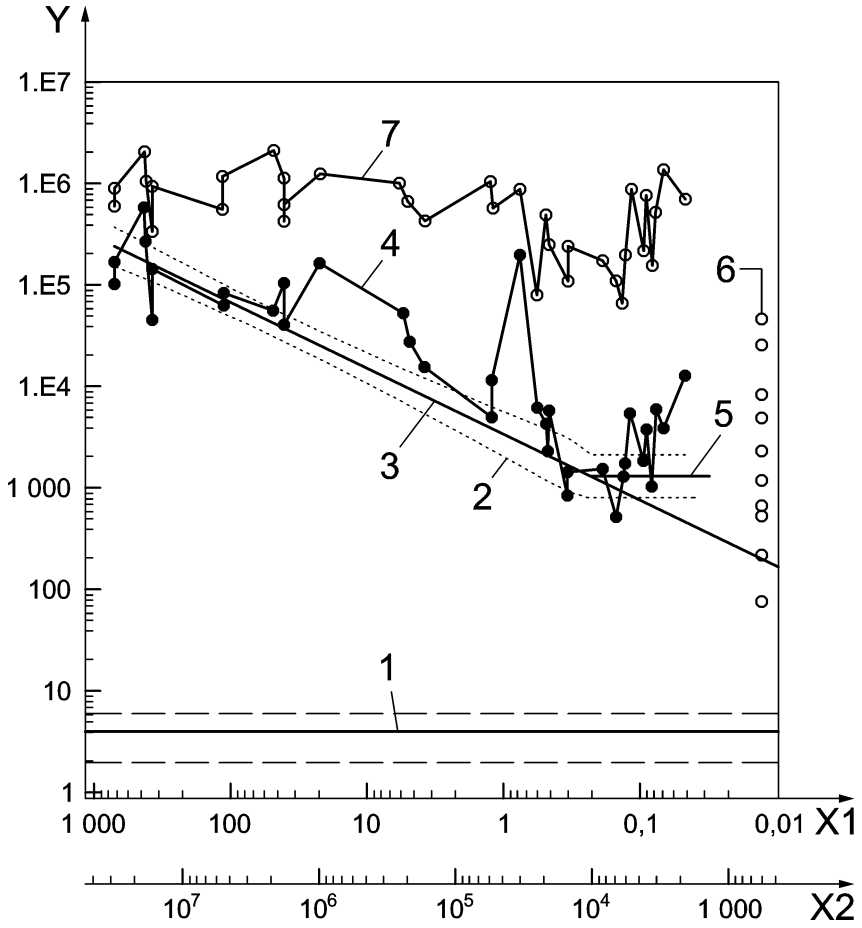
Figure 7 — Numbers of cases required for the Sigma-2 passive sampler [17]: Number of samplers required against rape pollen concentrations to be detected and pollen deposition for particular detection confidence levels, respectively

10.6 PMF

10.6.1 Sensitivity, detection limit and reproducibility

Results from the oilseed rape flowering period experiments using the pollen mass filter PMF are represented in Figure 8 in which the horizontal axes – on a logarithmic scale – denote the pollen concentration and pollen flux, respectively, from the GMO rape fields in accordance with the dispersal model. Pollen count in PMF samples is given on the vertical axis.

The total pollen counts in PMF samples were determined at levels between 100 000 and 2 million rape pollen grains per sample. In the case of rape pollen count, a declining trend from high values (top left) at more than 100 000 rape pollen grains per sample – which can be attributed to sites close to GMO rape fields – down to lower values (bottom right) can be observed, until a regional baseline level of approximately 1 000 rape pollen grains per sample is reached. Since both the rape pollen input originating from experimental fields with transgenes and from conventionally cultivated rape fields in the region are detected during pollen count analyses, individual sites in close proximity to other rape fields show higher values, accordingly. This was taken into account in the statistical data analysis of the results. Regression-analysis results demonstrate a statistically significant correlation ($P < 0{,}001$) between rape pollen count of PMF samples and the gradient of the dispersal model, thus providing evidence of the PMF's sensitivity. The reproducibility is reflected by the confidence band, whereas the detection limit of the PMF is represented by the horizontal line with confidence band. In more distant areas with less additional input from GMO fields, the curve of detected rape pollen counts sways in a "hockey-stick"-like way onto a level defining the baseline rape pollen input that corresponds to the density of the regional rape cultivation (horizontal line with confidence band). This baseline input in the area analysed was found to be at approximately 1 000 rape pollen grains/sample corresponding to a flux of 10^4 rape pollen grains/m^2 and a mean concentration of approximately 0,2 rape pollen grains/m^3. In the upper range of measurement, rape pollen counts from samplers in reference areas take a similar course, which is extended at the bottom by values from monitoring sites far away from rape cultivation areas, e.g. a site in the Bavarian Forest national park.



Key

1 detection limit

2 reproducibility (confidence band)

3 sensitivity (slope of regression line)

4 rape pollen

5 regional baseline level

6 rape pollen reference areas

7 total pollen

Y pollen count in PMF sample

X1 mean pollen concentration per m^3 air during exposure time T acc. to dispersion model (n pollen/m^3) at v = 1,93m/s

X2 pollen flux over exposure time T acc. to dispersion model (n pollen/(m^2 x T))

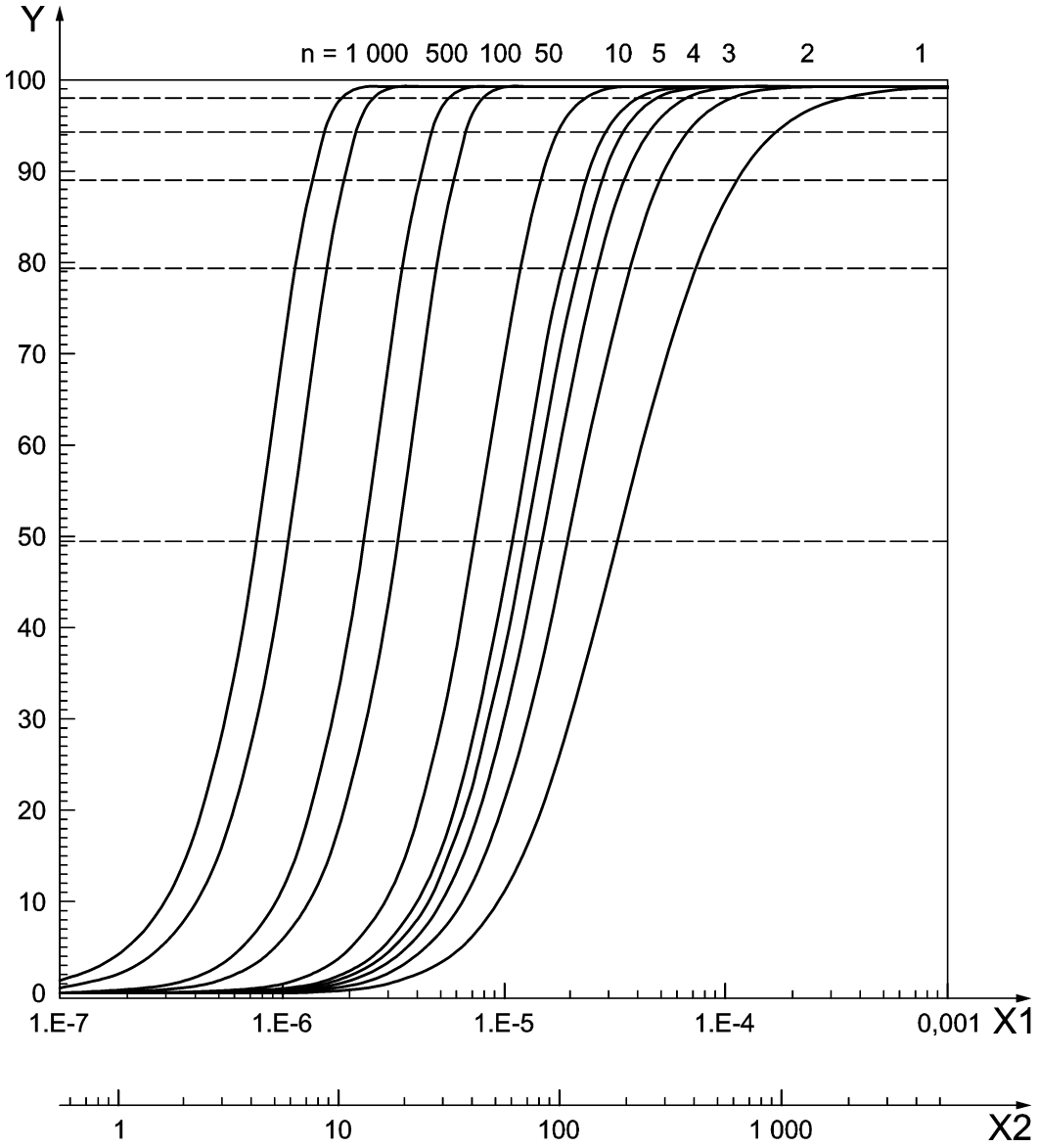
The lower axes indicate predicted values from the dispersal modelling for the mean rape pollen concentration (X1) over the exposure time T = 34 d and the respective pollen flux (X2). The vertical axis (Y) designates the pollen count per sample as measured with the PMF.

Figure 8 — Sensitivity, detection limits and reproducibility of the pollen mass filter PMF [17]

10.6.2 Detection confidence level and required numbers of cases

Figure 9 shows the detection confidence and concentration level of the PMF for rape pollen against pollen flux in various numbers of cases. The vertical axis represents the detection confidence level. Pollen flux (in rape pollen count/m^2) is displayed on the lower horizontal scale; the second scale refers to the mean rape pollen concentration as determined in dispersal models for the boundary conditions of the rape pollen experiment of 2001.

In pollen counting, the 50 % detection confidence level of the PMF is given at a pollen flux of approximately 200 pollen grains/m^2, corresponding to a mean pollen concentration of approximately 0,000 03 pollen grains/m^3 over 34 d, or approximately 0,000 16 pollen grains/m^3 over seven days, related to the experimental conditions of 2001. The 95 % detection confidence level of the PMF sampler is reached at pollen fluxes larger than 1 000 pollen grains/m^2, corresponding to a concentration of approximately 0,000 2 pollen grains/m^3 over 34 d or 0,000 8 pollen grains/m^3 over seven days. Detection limit and validity increase with increasing numbers of cases, in accordance with the curves shown.



Key

Y detection confidence level (in %)

X1 pollen concentration to be detected (*n* pollen/m^3) at *v* = 1,93 m/s

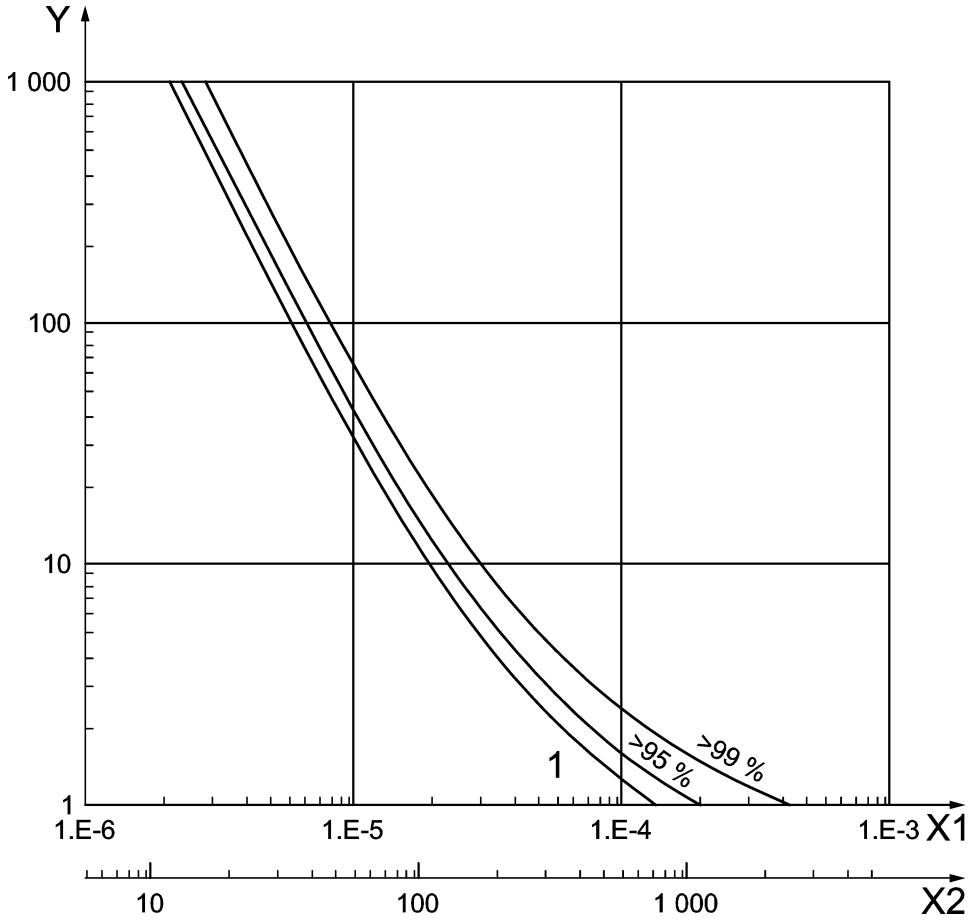
X2 pollen flux to be detected (*n* pollen/(m^2 x *T*))

Figure 9 — Detection confidence level of the PMF sampler for rape pollen against pollen flux and various numbers of cases [17]

To keep the detection limit and validity to a distinct level in a monitoring for all sites, this may be achieved for microscopic counting by increasing the subsample to be counted (dynamic counting, see 7.3.3) and – most important - for molecular-biological analysis by increasing the number of samplers per site according to the intensity of pollen flow expected at the sites. Figure 10 shows the number of cases required for GMO monitoring for chosen detection confidence levels. A subsample of being analysed microscopically has been taken here as one case unit. On the horizontal x-axis the detection limit is stated in concentration and flux units. The vertical y-axis denotes the number of cases that are required to keep a distinct detection probability indicated by the curves.

NOTE For general monitoring purposes, usually a subsample for counting is around 2 % to 5 % of the whole sample (see 7.3.3) to keep the majority for molecular biological analysis. For pollen type specific monitoring tasks a separation of the target pollen type can be undertaken and by this the whole sample can be analysed microscopically as well as molecular-biologically (see Annex A for maize pollen monitoring as example).

Figure 10 shows the number of cases required for GMO monitoring against the chosen detection confidence level.



Key

1 detection confidence level > 90 %

Y number of samplers

X1 mean pollen concentration to be detected (n pollen/m^3) at v = 1,93 m/s

X2 mean pollen flux to be detected (n pollen/(m^2 x T))

Figure 10 — Numbers of cases required for the passive sampler PMF [17]: Number of samplers required against rape pollen concentrations to be detected and pollen fluxes for particular detection confidence levels, respectively

10.7 Parallel measurements

The following coefficients of variation were obtained from parallel measurements carried out from 2001 through 2003 (Table 1 for the PMF and Table 2 for the Sigma-2 passive sampler).

Table 1 — Coefficients of variation for pollen flow rate obtained from parallel measurements with the PMF sampler with respect to total and pine pollen; survey carried out in 2001 to 2003 (Data taken from [8; 17])

PMF

Pollen flux rate		
	Total	**Pinus**
	$n/(m^2 \times d)$	$n/(m^2 \times d)$
Mean value	2 304 000	687 000
Minimum	133 000	41 000
Maximum	11 901 000	2 607 000
Coefficient of variation		
Mean	17,2 %	14,9 %
Stand. dev.	17,3 %	13,8 %
Minimum	0,4 %	0,6 %
Maximum	63,3 %	50,2 %
n	29	24

Table 2 — Coefficients of variation for pollen deposition rate obtained from parallel measurements with the Sigma-2 passive sampler with respect to total and pine pollen; survey carried out in 2001 to 2003 (data taken from [8; 17])

Sigma-2 passive sampler

Pollen deposition rate		
	Total	**Pinus**
	$n/(m^2 \times d)$	$n/(m^2 \times d)$
Mean Value	3 019 000	1 344 000
Minimum	77 000	13 000
Maximum	13 318 182	7 082 000
Coefficient of variation		
Mean	7,2 %	13,5 %
Stand. Dev.	8,7 %	10,5 %
Minimum	0,1 %	1,8 %
Maximum	34,0 %	37,7 %
n	18	18

The results for the total pollen and pine as frequent pollen type, which can be found at most locations and is present over a broad dose range, are contained. Comparable results were determined for other pollen types, such as rape and maize, however with smaller numbers of cases.

The coefficient of variation has an average value of 17,2 % ± 17,3 % with total pollen and 14,9 % ± 13,8 % with pine using the PMF.

With Sigma-2 passive sampler, the average value of the coefficient of variation is 7,2 % ± 8,7 % for total pollen and 13,5 % ± 10,5 % for pine.

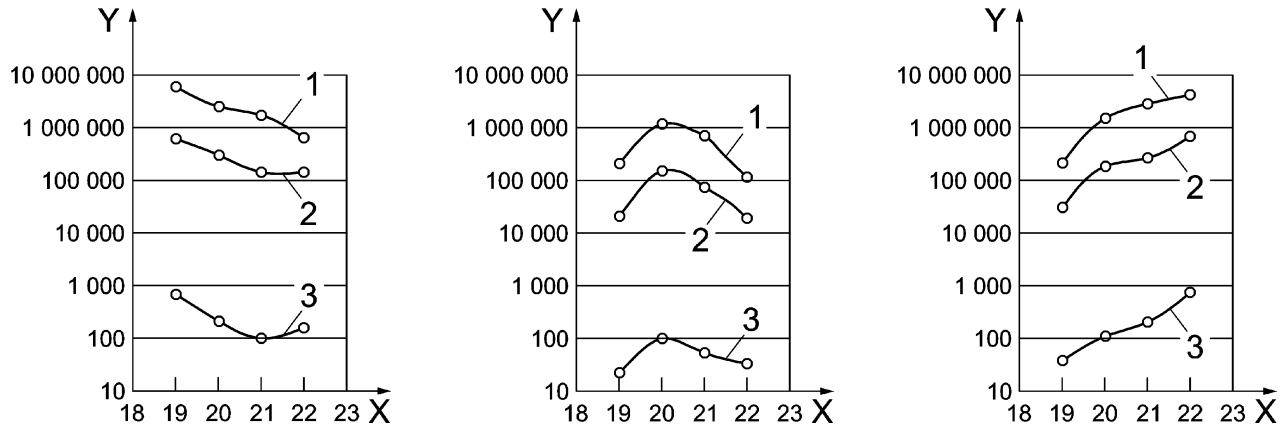
It is be taken into account that the accuracy in the quantitative determination of pollen depends on the value of the counted results according to the Poisson distribution, i.e. the error is relatively small with large quantities of pollen and it increases considerably with low numbers of pollen. In order to keep this error as small as possible, a dynamical counting is recommended when determining the quantity of the samples (see 7.2.3 and 7.3.3), which is geared to the observance of a sufficiently large count.

10.8 Comparative measurements using a standard volumetric pollen trap (Hirst type)

The results obtained from comparative measurements with the technical pollen samplers Sigma-2 passive sampler and PMF in 2003 with parallel pollen concentration measurements carried out using a volumetric pollen trap of Hirst-type[14)] [16] as a standardized active sampler of the pollen information services are shown in Figure 11. The trap samples continuously with an air volume rate of 10 l per minute equivalent to 14,4 m^3 per day.

The pollen was sampled on a weekly basis. For the pollen samplers Sigma-2 passive sampler and PMF, two devices were used simultaneously, for the Hirst trap one device. The figures show the sampling period (week) on the x-axis and the corresponding counted result in logarithmic representation on the y-axis; for the PMF as pollen flux, for Sigma-2 passive sampler as pollen deposition and for the Hirst trap as pollen concentration. The results for total pollen and pine and Poaceae as frequent pollen types were shown, because the counting error is negligible here (see 10.7). Comparable results for other pollen types exist. The standard deviations from the parallel measurements of Sigma-2 passive sampler and PMF are shown as vertical bars. The good correlation of the parallel measurements for Sigma-2 passive sampler and PMF results from the low standard deviations, which are partly smaller than the marking. With regard to the comparative measurements using the Hirst trap as reference devices, a good correlation of the three methods can be observed. This applies in particular when considering that concentration, deposition and flux depend on each other but are not identical quantities.

14) Trap type used: Automatic Seven-days spore and pollen trap, Burkard Manufacturing Co. Ltd., England. It is an example of a suitable product. This information is given for the convenience of users of this European Technical Specification and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.



Key

a) Y: Total pollen

X: Calendar week in 2003

1 mean pollen flow rate for PMF (n/(m² x d))

2 mean pollen count deposition rate for Sigma-2 (n/(m² x d))

3 mean pollen concentration for Burkard trap (n/m³)

b) Y: Pinus

X: Calendar week in 2003

1 mean pollen flow rate for PMF (n/(m² x d))

2 mean pollen count deposition rate for Sigma-2 (n/(m² x d))

3 mean pollen concentration for Burkard trap (n/m³)

c) Y: Poaceae

X: Calendar week in 2003

1 mean pollen flow rate for PMF (n/(m² x d))

2 mean pollen count deposition rate for Sigma-2 (n/(m² x d))

3 mean pollen concentration for Burkard trap (n/m³)

Mean values and standard deviation from parallel measurements for PMF and Sigma-2 passive sampler (two devices each, standard variation depicted by vertical bars, partly smaller than symbol); single measurements in case of Hirst trap. Exposure time: 10 d in weeks 18/19 (April 30th to May 9th, 2003), seven days in the following weeks (Data: [8; 17]).

Figure 11 — Comparison of PMF and Sigma-2 passive sampler with Hirst-type pollen trap used at monitoring site Westerloge in 2003

10.9 Pollen diversity

Technical pollen samplers detect air-borne pollen species.

When considering pollen dispersal, one shall distinguish dispersal by wind (anemophily) or by animals, e.g. insects (zoophily). Specific morphological features play a significant role in this respect, especially whether plant blooms produce nectar or not. If blooms do produce nectar, animals such as insects are attracted that provide dissemination of pollen (zoophily); if not, dispersal by wind (anemophily) is

preponderant. Even though classification of plant pollen is often based upon this distinction, and particular morphological features such as pollenkitt on pollen distributed by zoophily are observed at higher frequency, this distinction should be regarded in the sense of "more or less" rather than stringently. Rape pollen for example is often classified as being spread by zoophily, because the yellowness and nectar of blooms attract insects and pollen is coated with pollenkitt. In fact, rape pollen grains are intensively spread by both insects and the wind. Maize is regarded as a typical example of a plant whose pollen is spread by anemophily: Its blooms are nectarless and unattractive to insects, its pollen is without pollenkitt, and vast amounts of pollen are released during pollination. However, maize pollen grains are also collected by insects, e.g. by honey bees which use this pollen as an important protein source for the breeding of larvae in summer. For the aforementioned reasons technical and biological pollen sampling complement each other in GMO monitoring.

An important characteristic feature of pollen samplers used in GMO monitoring – especially with a view to future developments of GMO – is which pollen species can be detected with the technical sampler in the field. Elementary information and data on pollen input and blooming phases can be found at the pollen information services (e.g. www.polleninfo.org). Field experiments carried out so far have been carried out in various regions of Germany, other European countries and overseas. The pollen diversity of the technical sampler complements the spectrum found in the biological sample bee honey (see VDI 4330 Part 4 [32]) and gives rise to a total of more than 150 pollen species detected [17].

The results prove that detection with a technical sampler covers a wide pollen spectrum that extends significantly further than maize, rape, and sugar beet – the GMO currently of highest priority. Archived samples taken during pollen monitoring allow for retrospective analyses of further plant species that may become important in GMO monitoring at a later date.

11 Quality assurance and quality control

11.1 General monitoring strategy and terms of reference of pollen monitoring with technical samplers

The underlying monitoring strategy and the terms of reference of pollen monitoring carried out using technical samplers should be described.

11.2 Site protocol

Monitoring sites should be documented and protocols shall be compiled that provide:

— identification of monitoring site;

— exact position of sampling site to within an accuracy of 10 m, specification of elevation;

— surroundings of the monitoring station through topographical maps and – if possible – satellite or aerial photographs showing the general structure of land use: the immediate surroundings up to 1 km and the outlying areas up to 10 km;

— people responsible for execution of measurements, and individuals that can be contacted at site;

— date of installation of the monitoring station;

— photographical documentation of monitoring station and samplers.

If possible, fields in the surrounding area in which plants relating to target GMO are cultivated (e.g. rape, maize) should be marked on the maps.

11.3 Accompanying documentation for samples

The documents accompanying the samples shall provide the following:

— clear labelling of sample, from which monitoring site, sampler type, sampler number, and week of sampling can be inferred;

— date of sampling or change of sample (including time);

— person taking the sample, including phone number for enquiries;

— weight of PMF collection flask before and after rinsing for quantification of liquid volume used;

— comments on notable features at site, or with samplers or samples.

11.4 Parallel measurements

Parallel measurements should be carried out for at least 10 % of the sites.

11.5 Comparative measurements using active samplers as calibration bases

With a view to the pollen counts carried out with technical samplers, quality assurance also includes comparative measurements using standard active samplers (Hirst type pollen trap, Rotorod[15)]).[16)]

Ideally, the calibration bases should cover the range of pollen intensity and a range of pollen types. The sites should be located near cultivated areas for getting high enough values for calibration. Calibration bases can also be located outside the actual monitoring area. In doing so, however, a connection to existing permanent pollen monitoring stations equipped with active samplers is reasonable, and a technical pollen sampling should be carried out according to the same method as at the actual monitoring sites.

11.6 Quality assurance and reference materials

For quality assurance, the laboratories are obliged to regularly take part in matrix specific inter-laboratory ring tests.

For both the quantitative microscopic pollen analysis and the molecular-biological analysis of DNA (PCR) or proteins (ELISA), adequate reference material should be used.

In case of the microscopic pollen analysis this means mounted reference samples for any relevant pollen type under identical preparation conditions.

For the molecular-biological analysis (PCR, ELISA) adequate reference materials are needed for the different matrices (honey, pollen load, bee bread) with known numbers of pollen in respect to the target species.

As long as there are no certified reference samples commercially available, as an intermediate measure the laboratories should prepare internal standards in sufficient quantities that are used with each sample series.

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15) It is an example of a suitable product. This information is given for the convenience of users of this European Technical Specification and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

16) Authorization as a calibration basis can be obtained upon application to the advisory board via the chairman.

11.7 Qualification

When implementing procedures according to this Technical Specification the respective qualification of the individuals in charge shall be ensured.

Annex A
(normative)

Maize-specific requirements

A.1 Scope

This annex informs about maize specific requirements for GMO-pollen monitoring using the technical pollen sampling. It applies for all varieties of maize, genetically modified (gm) maize varieties as well as conventional ones. The method described in this annex has been optimized for detection of genetically modified maize pollen DNA by PCR for the task of a GMO-specific maize pollen monitoring. The requirements are shown by example of the Bt-maize MON810 authorized in the EU for cultivation.

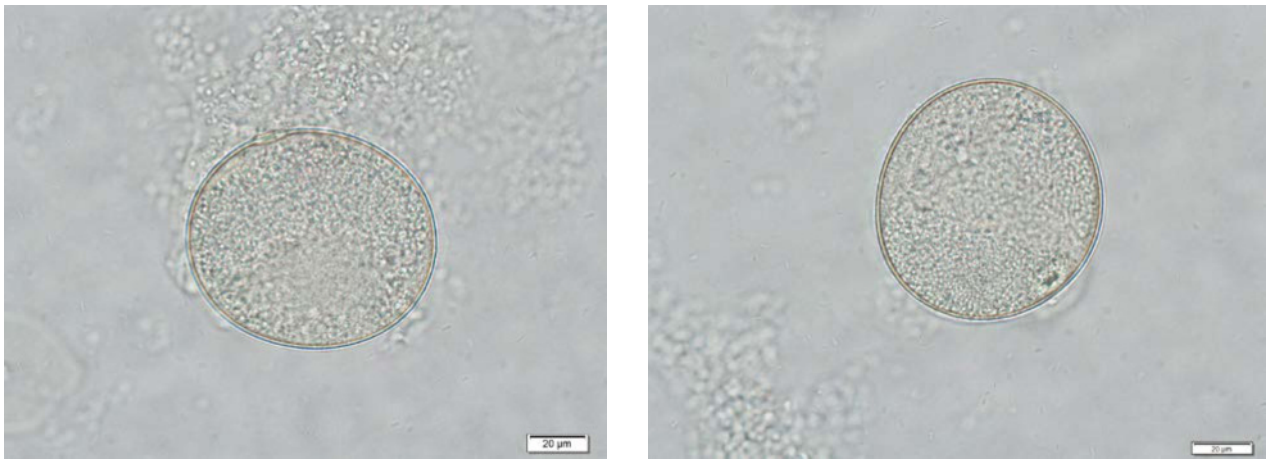
Main specific requirement: For quantitative maize pollen monitoring solely the PMF shall be applied for both the molecular-biological analysis of GMO as well as the microscopic pollen analysis due to the limitations of the Sigma-2 passive sampler to pollen smaller than 60 µm in diameter.

The aspects of molecular-biological analysis of GMO by PCR on transgenic DNA are specific for any pollen species and GMO-event and therefore, the analytical method shall be adapted and validated for any GMO specifically. This applies for GMO-screening purposes and multiple PCR aiming on more than one GMO, too.

A.2 Basic principles

Maize (*Zea mays*) belongs to the grass family (Poaceae) and is typically wind-pollinated (anemophil) producing enormous amounts of pollen. Depending on variety, growing and weather conditions, one plant generates on average ~25 million pollen (1 million to 50 million), summing up to 10^{11} to 10^{13} pollen per hectare [7; 12; 19]. Corn pollen is produced in the anthers in the male flower (tassel) at the top of the plant in about 2 m to 4 m height. The receiving female flower (ear) is located at much lower position at the stem and develops after fertilization to the cob. A typical ear has about 700 to 1 000 kernels with the silk as receptor organ for the pollen. Typically, maize pollen release takes place under warm and drying weather conditions for a period of seven to 14 days with the major portion of pollen being shed during midmorning to midday. Because pollen release may be interrupted by unfavourable weather conditions the period of pollen shedding in a field may last for three to four weeks and much longer in a region due to different maize varieties and growing conditions. Native to Central America (Mexico), maize is sensitive to cold and cultivation in Europe is limited to the warmer southern up to the temperate central regions of Europe. Whereas in subtropical countries and some Mediterranean parts of Europe maize might be grown nearly all over the year, in temperate climatic regions of Europe like Germany maize cultivation and flowering is restricted to the summer season. In Germany maize pollen are detected in the air commonly between end of June to end of August [21].

Maize pollen belongs to the greater and heavier pollen species with spherical to oval shape and a diameter of 80 µm to 120 µm on average (observed range: 60 µm to 125 µm) (see Figure A.1). The pollen is monoporate, the porus with operculum and a distinct anulus. The pollen wall is thin with scabrate exine structure. The cytoplasm is granular containing numerous starch granules. Dehydrated pollen vary in shape, resembling to an inflated ball. Maize pollen is typical in size, shape and structure and it can be distinguished from other grass pollen. The pollen of most wild grass species is smaller in size (<60 µm). Some grass cultivars like rye or wheat are similar in size but typically different in shape and structure.



a) **b)**

Figure A.1 — Maize pollen under microscopic magnification [Source: Katharina von der Ohe, LAVES Institute for Apiculture Celle]

A.3 Sampling

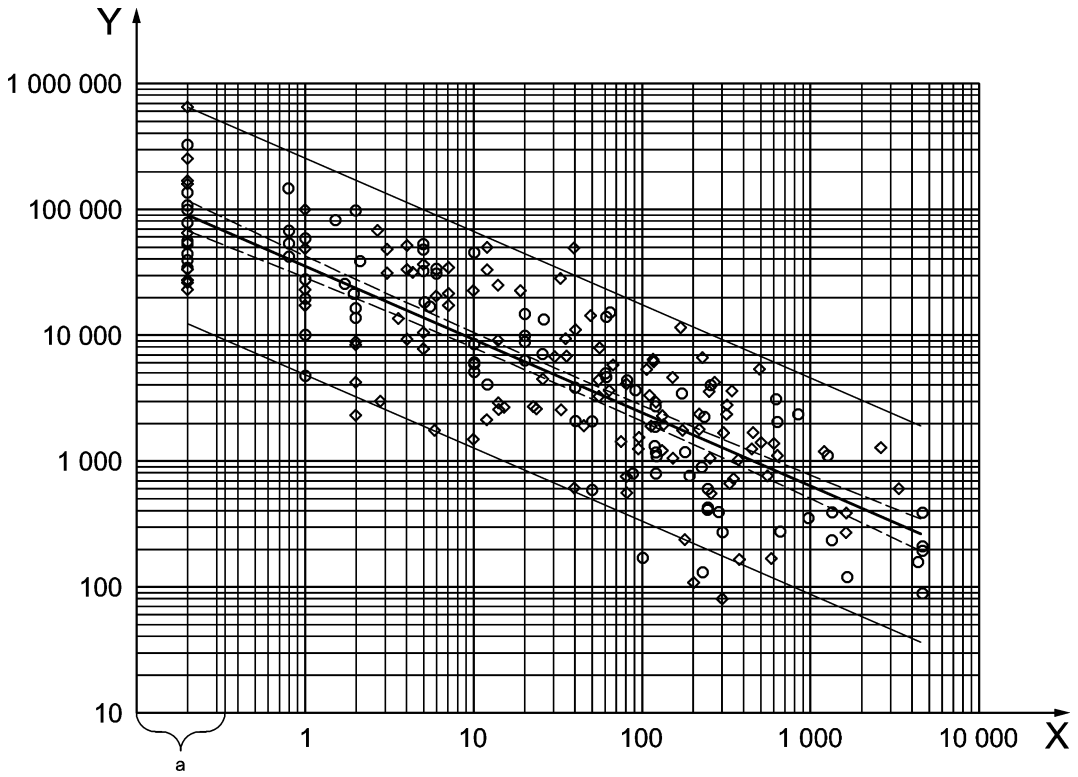
In case of maize pollen, the sampling shall be based on the PMF solely. The sampling procedure follows the general description in Clause 5.

The exact sample design depends on the particular task. In this annex an example is given. As outlined in 10.6, to ensure a valid detection of GMO by PCR, the detection limit of the PCR and the respective required minimum number of maize pollen in the sample is the critical parameter (see also A.6). According to 10.6, the sample design shall be geared to the expected maize pollen input at the various sample sites (required number of cases *k* of PMF sampler units per site). To increase the sample amount per site, up to four PMF units (*k*) can be stacked per pole under normal weather conditions[17)] (see Figure 2). For detection of GMO by PCR methods on a sensitivity level of 1 % gm (gm-maize/maize), a minimum of 1 000 maize pollen per sample as an empirical value should be aimed for. For best available technology, around 250 pollen are the limit [14].

For a most efficient sample design a validated maize pollen dispersal model shall be used for prediction of maize pollen input in the area (see, for example, [17]).

Without dispersal model, the expected amount of maize pollen per sample site can be estimated using the results of a regression analysis of maize pollen per PMF unit in relation to distance to the next maize field gained by long-term measurements shown in Figure A.2.

17) The PMF-duo with two units per pole has been proven to withstand extreme climatic conditions in over 3 000 m height at top of alpine regions.



Key

a within field

Y maize pollen counts per sampler unit PMF [*n*/PMF]

X distance to next maize field [m]

Figure A.2 — Maize pollen counts per PMF unit in relation to the distance from the nearest maize field:
regression analysis based on data gathered from 2001 to 2006 (circles [18]), rhombi refer to data obtained from 2007 to 2010 [21]

A.4 Sample preparation

Separation techniques shall be used to concentrate the maize pollen in the sample by separating the maize pollen from other dust and DNA-containing aerosol particles. The method described here has been shown to avoid inhibition and enables a sensitivity on a 0,1 % level of GMO-detection [14]. The preparation shall be proceeded uninterrupted to avoid still water conditions preventing germination of maize pollen and losses of DNA. Quality criteria for good performance is a recovery rate of intact maize pollen after preparation of higher than 95 % in the final sample.

Necessary steps of procedure:

a) The eight filter pads are each flushed for 10 s in beakers (Ø 90 mm) using 0,01 % Tickopur R30 solution[18)] in ultrasonic bath[19)]. Hereby, the filter pads are several times lifted out of the liquid using a long needle and then being transferred to a second beaker, repeating the procedure.

18) Dr. H. Stamm GmbH; Tickopur R30 is manufactured by the supplier mentioned above. It is an example of a suitable product. This information is given for the convenience of users of this European Technical Specification and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

b) The liquid together with the aerosols is transferred into a vacuum filtration unit (50 mm diameter PE-membrane filters 12 µm) specially equipped with a vacuum cascade filter unit[20)] as pre-filter containing PA-filter ronds (50 mm diameter) with mesh sizes of 125 µm (on 1 mm support filter rond) and 64 µm (on 1,000 µm support filter rond) as pre-filters for separation of the maize pollen.

c) Than the remaining content of the 1,5-l collection flask is transferred to the same vacuum filtration (b) avoiding any shaking until a small amount of liquid of ~100 ml remains. Than the flask is shaken and treated during 30 s in ultrasonic bath and, after shaking it again, the content is transferred to the vacuum filtration with post-rinsing.

d) The filter ronds and the membrane filter are stored in 50 mm-petri dishes and labelled stating sample and mesh size. They are inspected under the binocular and photographed for documentary purposes.

e) The 60 µm filter rond retains the fraction of 60 µm to 125 µm with the maize pollen sample, which is used for the further analytical steps referring to the maize pollen monitoring.

f) The other filter rond and the membrane filter containing the remaining particle fractions can be used for further analytical tasks, e.g. microscopic analysis of the pollen and spore diversity. The membrane filter of 12 µm contains the aerosol particle fraction of 12 µm to 60 µm. Most of other pollen species and spores as well as inorganic particles fall into this category. Some pollen and bioaerosol particles are larger than 125 µm, they are found on the filter rond 125 µm.

g) The maize pollen sample on the filter rond 60 µm can be 1) analysed immediately or 2) stored for later analysis:

   1) for immediate analysis see A.5 and A.6;

   2) storage of the filter rond with the maize pollen sample can be done air-dried (for 24 h at room temperature) or frozen at −18°C, either in the closed petri dishes or by transfer of the filter rond in a 50 ml tube by bending the rond slightly.

A.5 Quantitative microscopic pollen analysis

Quantitative microscopic analysis for maize pollen can be performed (a) on the filter rond directly or (b) in pollen suspension.

a) Direct microscopic counting of the maize pollen sample on the filter rond in the petri dish can be done using a high-resolution binocular under 80x-magnification equipped with a cross table or using a microscope with adapter for petri discs with diameter 55 mm. Identification of maize pollen is possible immediately after preparation when the rond is not any more soaked with water and the pollen are still not dehydrated, or, in case of dried samples, after slight moistening of the filter rond putting a film of water in the petri dish, so that the maize pollen is expanded in full shape. As the maize pollen are not distributed evenly on the filter rond, the counting shall be done completely or in a representative way. For efficient counting, the filter rond may be oriented on the cross table in such a way, that the fibres of the mesh are parallel to the x-y-axis of the cross table giving. The

19) Sonorex Bandelin Super RK 102. It is an example of a suitable product. This information is given for the convenience of users of this European Technical Specification and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

20) Vacuum-Cascade-Filter VCF, TIEM technic GbR. The VCF is manufactured by the supplier mentioned above. It is an example of a suitable product. This information is given for the convenience of users of this European Technical Specification and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

supporting filter of size 1,000 µm gives paths of 1,000 µm (open area) respectively 1,515 µm (including wire). The counting can now be done along the rows (x-axis) defined by the paths of the mesh grid. Depending on the number of maize pollen in the sample, the whole sample shall be counted or a representative portion, e.g. every second or third row. The inner diameter of the vacuum filtration unit is 46 mm, the respective total deposit area on the filter rond is 1,662 mm^2.

b) Pollen suspension: The maize pollen sample on the filter rond transferred into the 50 ml tube can be analysed in the suspension prior to DNA extraction (see A.6.2). From the 10 ml pollen suspension aliquots of 10 µl to 50 µl are taken for quantitative microscopic analysis. The dynamic counting method shall be applied guaranteeing a sufficient number of maize pollen counts (see 7.3.3).

A.6 Molecular-biological analysis of maize DNA using PCR

A.6.1 General

For detection of GMO the molecular-biological analysis of DNA using real-time PCR is described here. The method has been successively developed and validated for maize [8; 14; 17; 20], the description here reflects the current state (February 2013).

A.6.2 DNA extraction

a) The maize pollen sample on the filter rond > 60 µm transferred in a 50 ml centrifugal tube is used.

b) 10 ml of Cetyltrimethylammonium bromide (CTAB) buffer (13,3 g/l CTAB, 0,93 M NaCl, 66 mM Tris-HCl, 13 mM Na_2EDTA, pH 8) is added and by thoroughly shaking the maize pollen are removed from the filter rond. The filter is taken out and inspected visually under the binocular for complete removal of maize pollen. The maize pollen can now be counted in the suspension microscopically (see A.5 b)).

c) Glass beads (1 mm glass beads) and an appropriate volume of the suspension is vortexed twice for one minute at 1 400 × g keeping the suspension on ice for a 30 s interval between the mixes, and finally incubated overnight at 60°C in the presence of Proteinase K (0,33 mg/ml).

d) After centrifugation at 5 000 × g for 10 min, the supernatant is transferred to a new tube and extracted with 0,9 volumes of chloroform. DNA is then precipitated with 0,8 volumes of isopropanol at room temperature for 30 min.

e) After centrifugation at 11 000 rpm for 15 min the pellet is washed twice in ethanol 70 %, air-dried and re-suspended in 60 µl to 80 µl of sterile water.

NOTE The same procedure is followed for DNA purification from pure maize pollen (from 10 mg to 100 mg of maize pollen). In this case, an RNase treatment is added at the end (RNase 5 ng/µl) for one hour at 37°C. After RNase treatment, DNA is ethanol-precipitated and re-suspended in 60 µl to 80 µl of water.

f) The DNA concentration of all DNA extracts is measured by fluorimetric means.

A.6.3 Real-time PCR analysis

All real-time PCR analyses are preferentially performed applying validated methods according e.g. to the ISO Standards or Codex guidelines. A listing of validated methods for GMO analysis can be found in the “Compendium for Reference Methods in GMO analysis” [25]. For the detection and quantification of gm pollen, methods should preferentially comply with the performance criteria set by the European Network of GMO Laboratories.

A positive control (PC) and a negative control (NC) pollen sample are to be included at all times in the screening, the identification and/or quantification analyses.

All PCR on airborne samples are to be analysed at least in duplicate and in case of conflicting results (one positive, one negative) a third measurement shall be performed.

PCR inhibition analysis should be performed by determining the PCR efficiency of the extracted DNA over a fourfold serial dilution range using an appropriate marker gene (e.g. an endogenous species marker of the pollen under investigation).

A.7 Determination of the target parameters for GMO monitoring and assessment of the results

For the task of GMO monitoring relevant to this Technical Specification, the results of the PCR shall be reported as percent gm-maize pollen to total maize pollen (GM-maize DNA to maize DNA). The number of maize pollen counts per sample and per single PCR aliquot shall be included.

For GMO monitoring purposes false-negative results are a critical issue. The assessment shall be undertaken to exclude any GMO input at the site as testing hypothesis. The results shall be presented in such a way that the validity can be seen and conflicting results are documented in a reproducible way. The assessment on GMO shall lead at least to three classes:

— “GMO input detected [2+]” in case of consistent positive measurements; in the example here: two;

— “GMO input cannot be excluded [2-, 1+]” in case of conflicting results; for example: two negative and one positive measurements;

— “GMO input not detected [2-]” in case of consistent negative measurements.

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