PD CEN/TS 16817-2:2015

BSI Standards Publication

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

Part 2: Biological pollen sampling using bee colonies

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National foreword

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Außenluft - Monitoring der Wirkungen von gentechnisch veränderten Organismen (GVO) - Pollenmonitoring - Teil 2: Biologische Pollensammlung mit Bienenvölkern

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European foreword

This document (CEN/TS 16817-2:2015) 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*;

— *Part 2: Biological pollen sampling using bee colonies* [the present document].

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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 [6; 7]. 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). Hence, a monitoring procedure that involves recording and documentation of input and distribution of GMO via pollen in a monitoring network mirroring the natural environment is required. For this, technical (CEN/TS 16817-1) and biological sampling of pollen as well as PCR-screening (polymerase chain reaction) procedures are employed to provide evidence of GMO-exposure. The biological sampling system using honey bee colonies is described in the present Technical Specification.

VDI/Guideline 4330 Part 1 [3] presents the necessary fundamentals for the understanding of this Technical Specification. The sampling of pollen in the sample matrices honey, pollen load and bee-bread [5] needs to be viewed in conjunction with the technical sampling for the GMO-monitoring [4].

The use of the biological, actively foraging honeybee and the technical passive samplers complement each other in a manifold and positive way for pollen monitoring of GMO. Therefore it is reasonable to use both. The technical sampling (CEN/TS 16817-1) is based on stationary point-samplers [1]. They give a record of pollen exposure in the air at the sample site that correlates with the prevailing wind direction and relative position to the surrounding pollen sources. The biological sampling using honey bee colonies serves as indicator for GMO exposure in an area and for exposure to roaming insects. Bees display a spatially averaging sampling activity, which represents a cross section of the established, blossoming plants in the area according to the bees collection activities. A wide spectrum of pollen species is recorded using both sampling methods with the procedures complementing each other across the vegetation period [21].

1 Scope

This Technical Specification describes a procedure through which pollen – in particular pollen of genetically modified organisms (GMO) – can be sampled by means of bee colonies.

Bee colonies, especially the foraging bees, actively roam an area and are therefore area related samplers. Pollen sampling depends on the collection activity of the bees and the availability of pollen sources within the spatial zone according to the bees' preferences (supply of melliferous plants). A colony of bees normally forages over an area of up to 5 km radius (median 1,6 km, mean 2,2 km), in rare cases some bees may also forage in greater distances up to 10 km and more [26].

Foragers fix the gathered pollen on the outside of their hind legs (pollen loads, also known as pollen pellets). Inside the hive they place these pollen loads into comb cells close to the brood nest (bee bread). Furthermore, foragers gather nectar and honeydew. Nectar contains pollen which fell from the anthers of the blossom into the nectar drop, or pollen which was dispersed by the wind and sticks in the nectar of other blossoms or adheres to the sticky honeydew of plants. Nectar and honeydew are converted to honey and stored by the bees in the beehive.

Honey, pollen load and bee-bread may be used as sample matrices for the subsequent analysis of pollen as it is possible to concentrate sufficient amounts of pollen for microscopic and molecular biological diagnostics.

Microscopic analysis is used to identify the various pollen types and to quantify the exposure to the target pollen types in question. GMO exposure is analysed by molecular-biological methods: For analysis of pollen DNA quantitative PCR methods are used and described here in this Technical Specification. The analysis of GMO specific proteins and toxins in pollen is possible, too, using ELISA, but to this date the method has not been evaluated enough in pollen matrices for standardization in this Technical Specification.

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.

DIN 10760, *Analysis of honey — Determination of the relative frequency of pollen*

3 Terms and definitions

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

3.1
bee bread
pollen load stored in comb cells close to the brood nest

3.2
bee colony
colony of the honeybee species *Apis mellifera*

3.3
beehive
hive
container in which honeybees are kept by beekeepers

3.4
event
<genetics> unique DNA recombination event that took place in one plant cell, which was then used to generate entire transgenic plants

3.5
flying bee
foraging bee
forager
worker bee of a colony which is active outside the hive

3.6
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 [6], modified — The content of the definition was changed.]

3.7
honey
product generated by bees from the raw materials nectar and honey dew

3.8
honeydew
sugar containing secretion of aphids and cicadas sucking on plants

3.9
melliferous plant
plant from which nectar, honey dew and/or pollen is offered as sources of food for bees

3.10
monitoring
environmental monitoring
characterizing the state and quality of the environment and its changes by measurements/observations in regard to defined objectives

3.11
nectar
sugar containing secretion of the nectar glands in or from blossoms

3.12
pollen
male gametophyte of the flowering plant

3.13
pollen and honey flow
food supply within the environment (foraging area) of a bee colony

3.14
pollen load
pollen pellets
pollen brought into the bee colony by the pollen foraging bees at their hind legs

3.15
pollen type
pollen species
class of pollen being distinguished by microscopic means on species, family or other order level

3.16
sampling
pollen sampling
collection of particles, here pollen by technical or biological means

4 Basic principle of the procedure

The bee colony serves as biological active sampler of pollen. The bee colonies are positioned within the area under investigation i.e. relocated bee colonies are used or bee colonies which are already present, i.e. permanent apiaries by local beekeepers.

Flying bees forage for food sources (supply of melliferous plants) and if successful, bring in the raw materials nectar, honey dew and pollen. By gathering nectar, honeydew and pollen, bees collect a fraction of the pollen present at the time in the area. These pollen are stored in wax combs as honey and bee-bread and are available for future analyses. Further on, the collected pollen load of the bees may be used directly as sample matrix gained by pollen traps at the hive entrance. Advantages and disadvantages of the different matrices are given in Clause 5.

Depending on the supply from melliferous plants, if there is a shortage bees also gather pollen from anemophilous plants. Bees also require water which they collect from numerous sources (dew, open bodies of water, etc.). Pollen is produced in the anthers of the flowers. Anthers burst apart after reaching maturity, making pollen available. Pollen, released from the anthers of the same flower, also stick to the nectar of this flower. Anemophilous pollen is distributed by wind and can stick to honeydew or nectar. So anemophilous pollen can be collected indirectly by the bees as well as by flying through the air.

The area used by the bees depends on various factors (weather, availability of melliferous plants, utilization of landscape and landscape structure, etc.). The main foraging distances are [26]: modal distance from hive to forage site 0-0,7 km, median distance 1,6 km, mean distance 2,2 km, maximum 10 km.

Exposure time may be flexibly specified from a minimum of five days up to several weeks. For exposure times of more than a week, sampling in intervals is also possible.

The pollen samples are analysed using light microscopy (palynology) and by molecular biological analysis (e.g. PCR).

5 Sample matrices

5.1 Honey

Honey is produced by bees from the gathered raw materials nectar or honeydew and is stored in special combs (honeycombs). Both raw materials contain pollen among other things. Centrifuges extract honey from the honeycombs.

Honey yield is more reliable than bee-bread or pollen load and is thus clearly preferable. The available amount of pollen load and beebread depends a lot more on the supply of plants and the consumption by the bees. The matrix honey is significantly better suited for light microscopic and molecular biological analyses [10; 25; 32]. But the amount of anemophilous pollen grains in honey is small.

For comparative studies, extracted honeys are preferable to the other matrices (pollen load and bee bread) as a strong homogenization occurs from the type of extraction. Extracted honey possesses a better spatial and time representation. Basically, two sampling dates per bee site may be assumed for each region (spring and summer honey). Summer honey is not collected by all bee-keepers regularly.

5.2 Pollen load

Pollen load is the pollen brought in separately by the foraging bees. Pollen load can be taken off the hind legs of homecoming bees using special pollen traps. Pollen traps are devices which can be attached to the front of the hive (front pollen trap) or inside between bottom and first hive body (inside pollen trap). These traps have a hole pattern. Passing through these holes incoming pollen foragers will lose their pollen load. The removed pollen loads will drop in a collection vessel. Using pollen traps negative effects on foraging behaviour can occur. These effects are less when using inside pollen traps than front pollen traps.

Pollen load has advantages over honey under specific circumstances: differentiations of flowering and foraging time, foraging of nectarless melliferous and anemophilous plants. However, to implement these advantages, many more sampling dates and efforts are necessary. Molecular-biological analysis of the matrix pollen load needs more preparatory steps than the matrix honey. Advantage: greater amounts of pollen.

5.3 Bee-bread

Bee-bread is the pollen brought in separately by the bees which is stored in special areas of the combs. Bee-bread may be extracted by cutting out corresponding areas of the combs.

Bee bread has advantages over honey under specific circumstances: differentiations of flowering and foraging time, foraging of nectarless melliferous and anemophilous plants. To implement these advantages, many more sampling dates and efforts are necessary though. PCR analysis of the matrix bee bread is much more complicated than of the matrix honey. In addition, bee bread is sometimes not available due to consumption by nurse bees.

6 Sampling procedure

6.1 General

For the sampling procedure including site conditions, placing the colonies and sampling the pollen matrices the “Good Beekeeping Practice” shall be regarded (see Annex C). Some general aspects and specific requirements in the scope of this TS for GMO-monitoring are stated here.

6.2 Bee colony and hive

The bee colony includes the hive (box in the broader sense as housing for the bees), frames with wax combs, a queen, 10 000 to 40 000 worker bees as well as several hundreds of drones in certain months. It is managed by the beekeeper according to good beekeeping practice (see Annex C).

Modern hives are multiple-storey hives (one or up to five storeys or bodies) made of wood or Polystyrene (PS) foam with wooden frames and mostly wax foundation. These types of hives are predominant. Within the frames honeybees build their combs. Due to the type of hive number and size of the frames are different. According to good beekeeping practice size of multiple-storey hives can be adapted to the size of the colony or the space needed by the colony.

6.3 Sample site

At least one bee colony is positioned at a fixed site. Exact positioning takes place according to good beekeeping practice (protection against flooding, storm, branch lashing, etc.) [22; 33]. Regular attendance to the colony of bees needs to be guaranteed (approximately every 9 d to 14 d).

6.4 Preparation and assembly

No further activity is required regarding the assembly of previously installed colonies of bees (permanent sites).

Newly migrated bee colonies shall be placed at the specified site. Migration shall take place outside the flying times of the bees from late in the evening till early in the morning. The previous site shall be at least five kilometres away from the new location in order to exclude a return flight of the bees back to the old location.

Stationary as well as migrating bee colonies should be harvested beforehand when there is a surplus of honey (more than required for bees' needs).

6.5 Exposure time

Exposure time shall be defined depending on the task of the monitoring. For example, should the pollen distribution of a plant species such as oil seed rape be recorded, it is reasonable to define the exposure time covering the flowering period, e.g. at least from beginning of the flowering (5 % to 10 % open blossoms, BBCH code 61 [18]) until withering of the last blossoms.

For newly placed bee colonies, exposure starts after putting up the bee colonies. After approximately two days, the foraging bees have explored the area and are familiar with the local environment. After five days at the earliest, the first samples of honey could be taken of combs.

Depending on honey flow larger amounts of honey may be extracted after approximately two weeks or later by removal of entire combs.

Where there is insufficient supply of food sources with long exposure times, honey or bee bread placed in storage might be consumed by the bees.

The colonies shall be regularly attended during longer exposure times (more than nine days) according to good beekeeping practice [22; 33 and Annex C].

6.6 Sampling dates

Sampling dates are to a large extent defined by the exposure time (see 6.5). For longer exposure times samples may be taken at intervals.

For the matrix honey, based on the amount available, either pieces of comb or entire combs may be removed.

For pollen loads: Front pollen traps (see 5.2) have a significant effect on foraging behaviour. Pollen load can only be taken from one colony for a short time (e.g. one day). If longer exposure time is necessary, more colonies should be placed at the site so one colony after the other can be used for pollen collection. This can be avoided by using inside pollen traps that has less effects. They are therefore better suited to cover a flowering period by daily sampling without intervals.

6.7 Extraction, transport and storage

Complete honeycombs are stored inaccessibly for bees after removal and, following completion of the necessary beekeeping tasks, are immediately taken to the bee-keeper's apiary for extraction according to good beekeeping practice.

Alternatively honey can be obtained by scraping out of honey combs (preferably capped, ripe areas). A representative sample (at least 500 g) of the stirred honey of all bee colonies at one site is sealed in a jar and kept cool (<8°C). For further analysis, the honey is frozen on arrival at the laboratory (<-18°C).

At the extraction site, the pieces of comb for bee-bread are placed in sufficiently large containers or plastic bags carefully sealed and then labelled. In a cold chain (<8°C), samples are delivered to the laboratory and frozen for further processing (<-18°C).

If possible, each day the pollen load should be taken out the traps and transferred in sufficiently large containers or plastic bags carefully sealed and then labelled. In a cold chain (<8°C), samples are delivered to the laboratory and frozen for further processing (<-18°C).

7 Palynology

7.1 General

In principle, extracted honey is the sample matrix to be preferred (see Clause 5) for primary pollen types that are abundantly collected and present in honey. For some other pollen types relevant for GMO-monitoring, like for example maize, pollen loads are preferably used. In case of non-primary pollen types that are underrepresented in the samples an up-concentration may be advisable by using separation techniques [for example see Annex A for maize pollen].

7.2 From sample preparation to embedded slide preparation

7.2.1 General

The sample *S* is the amount of matrix collected by a bee colony during the collection period. The total sample defines the amount collected over the whole flowering season.

7.2.2 Honey

From the collected honey sample *S* a subsample *U* of 5 g of honey dissolved in 10 ml distilled water is centrifuged [DIN 10760; [20]].

Excess liquid is thoroughly rinsed off on absorbent paper. 0,5 ml distilled water is added to the sediment (in the centrifuge tube), while it is thoroughly stirred. An aliquot *A* with 0,1 ml of the suspension is spread evenly over the marked area of 22 mm × 22 mm (mark off area beforehand) of the slide. The slide is dried for approximately one hour and then immersed in glycerol-gelatine (size of the cover slip 22 mm × 22 mm; see DIN 10760).

For molecular-biological analysis the sample preparation is described in Clause 8.

7.2.3 Pollen load

The pollen load should be cleaned from other material like leaves, etc. which have fallen in the pollen traps.

For each daily pollen load the weight is determined.

For representative analysis the sample shall be homogenized. The pollen load is diluted 1:1 (w/w) with distilled water and stirred for approximately one hour until total homogenization (magnetic stirrer).

For quantitative microscopic pollen analysis an aliquot *A* with 15 µl of this solution are transferred onto a microscopic slide, 30 µl of distilled water added, homogenized again and spread over an area of 22 mm × 22 mm. After drying on a warming plate at 40°C embedding takes place with the mounting medium Kaiser's Glycerine jelly and a 22 mm × 22 mm cover slip.

For molecular-biological analysis the sample preparation is described in Clause 8.

7.2.4 Bee bread

The bee-bread is extracted from the pieces of honeycomb (10 cm × 10 cm) with a spatula. Subsequently bee-bread is diluted 1:1 (w/w) with distilled water and stirred for approximately two hours until total homogenization (magnetic stirrer).

An aliquot *A* with 15 µl ml of this solution is transferred onto a microscopic slide, 30 µl of distilled water added, homogenized again and spread over an area of 22 mm × 22 mm. After drying on a warming plate at 40°C embedding takes place with the mounting medium Kaiser's Glycerine jelly and a 22 mm × 22 mm cover slip.

For molecular-biological analysis the sample preparation is described in Clause 8.

7.3 Microscopic analysis

Light microscopic analysis for honey takes place according to DIN 10760 in the laboratory [9; 19; 23; 24; 29; 30; 31]. The procedure for the other matrices is analogous. Analysis takes place at 400-times magnification. In a first general check, quality of the microscopic mount and in particular the homogeneous distribution of the pollen is tested. The pollen should be identified by the established literature for determination [19; 29; 31; 32].

Microscopic analysis is done mainly at 400-times and in some cases at 1 000-times magnification:

Pollen grains of every 5th row (cross slide) are identified and counted for each pollen type present to not less than 1 000 pollen grains in the total sum.

In case that for any target pollen type less than 5 counts have been detected, then successively another 6 rows shall be counted (only the targeted pollen type) until either 5 counts have been reached or the whole sample has been analysed (dynamic counting method).

The total pollen counts over all pollen types are stated as well as the pollen counts for the target pollen type(s) *i* in question.

First the sum of pollen counts of each pollen type *i* is extrapolated to the sample aliquot on the mount by taking the ratio of the number of counted rows (counted area) to the total number of rows (total area of the cover slide on the mount) into account. The number of rows may vary depending on the microscope (depends on the size of field of vision. The count to at least 1 000 pollen enables a statement on a detection limit of 0,1 % (detecting 1 pollen).

$$N_i = \text{sum pollen counts} \times \frac{\text{total rows}}{\text{counted rows}} = \sum_{k=1}^{z} n_{i,k} \cdot \frac{K}{k} \left[n \text{ pollen} \right] \qquad (1)$$

where

N_i	is the number of pollen counts of type *i* on mount;
$n_{i,k}$	are the pollen counts of type *i* in row (*k* = 1, 2, 3, …., *K*);
i	is the pollen type;
k	is the index of row on microscopic mount (*k* = 1, 2, 3…, *K*);
K	is the total number of rows on microscopic mount;
z	is the number of counted rows.

7.4 Pollen diversity

The pollen diversity for each sample site and matrix shall be noted in form of a table. The results may be presented qualitatively (detected) or quantitatively. Quantitative results are required for at least the target pollen types and total pollen content.

8 Molecular-biological analysis

8.1 General

Molecular-biological analysis is used for identifying GMO-exposure.

The analysis of the DNA may be done using PCR (Polymerase-Chain-Reaction). Here the state of the art is given by quantitative PCR methods, even though the results of environmental samples – especially in case of pollen samples – may often be restricted to be semiquantitative. For optimal results in PCR analysis the sample preparation shall be performed specifically for any pollen type and matrix. Further screening methods for more than one GMO may be used, too, which are, in general, less sensitive. For the molecular-biological analysis of pollen DNA using PCR a sufficient high amount of target pollen is necessary to meet the detection limits. For performing the analysis with a detection limit of 0,1 % GM-content a number of at least in the order of 10 000 pollen of the target pollen type is required. Therefore, the counting should precede the PCR analysis.

The rationale behind this is that for PCR analysis the sample gets divided into aliquots and that any single aliquot needs at least 3 DNA copies of the target GMO to be detected. 0,1 % detection limit of the target GMO means 1 GM-pollen out of 1,000 pollen of that pollen type. Each pollen contains 2-3 DNA copies. The extraction efficacy of pollen DNA depends on the pollen type and extraction method used and ranges between 10 to 50 % for are commonly good performance. Per single PCR-analysis only a portion, between 5 to 10 % of the extracted DNA (aliquot) is used, this allowing repeated analysis and / or the analysis of more than one event/GMO. Considering the Poisson distribution for the probability of at least 1 extracted target DNA copy in a single aliquot, this results in a minimum pollen content in the magnitude of 10 000 pollen of the target pollen type in the sub-sample taken for the PCR. For screening methods like the analysis of the promoter 35S the calculation is the same taking here the minimum percentage into account to which a GM pollen type shall be present in the sample to be detected.

NOTE GMO are usually single copy events.

After DNA-extraction, various methods of quantitative PCR are possible. By the state of the art this includes single event analysis to multiple event detection. An example for Bt-maize is given in Annex A.

Some GMO express specific proteins like for example the Bt-toxin, that can be analysed using ELISA (Enzyme-linked immunosorbent assay). This method will not be described further in this Technical Specification.

In the following the general sample preparation steps are given which are necessary independently of GMO and pollen type and prior to the specific preparatory steps.

8.2 Sample preparation

8.2.1 Honey

The amount of honey used for the analysis depends on the pollen counts detected in 7.3 for the respective pollen types questioned in regard to keep detection limits.

EXAMPLE A honey sample shall be tested specifically for GM-oil seed rape. The quantitative microscopic pollen analyses resulted in 1 300 Brassica pollen per g honey. For the GM-specific analysis the required minimum sample amount to achieve 10 000 target pollen would be > 50 g and > 200 g for optimal performance.

In case of non-primary pollen types with frequencies H lower 10 %, for optimal performance of the PCR the target pollen types shall be concentrated by using separation techniques. An example is given in Annex A for maize pollen shown with the matrix pollen load.

8.2.2 Pollen loads

The homogenized daily pollen load samples from 7.2.3 are splitted in two halve parts.

One part (A) is retained for any potential day specific analysis.

The second part of all daily pollen samples are combined to one pooled sample B for overall analysis. The pooled sample shall be homogenized using a magnetic stirrer or mixer.

Halve of the pooled sample B1 (25 % of the original sample) containing all pollen types will be stored as retain sample and for any potential future screening purposes.

The other halve B2 will be further prepared for GMO-/pollen type specific analysis.

In case of non-primary pollen types with frequencies H lower 10 %, for optimal performance of the PCR the target pollen types shall be concentrated by using separation techniques. An example is given in Annex A for maize pollen shown with the matrix pollen load.

8.2.3 Bee bread

The homogenized bee bred sample from 7.2.4 is splitted in two halve parts.

One part B1 (50 % of the original sample) containing all pollen types will be stored as retain sample.

The other halve B2 will be further prepared for GMO-/pollen type specific analysis.

In case of non-primary pollen types with frequencies H lower 10 %, for optimal performance of the PCR the target pollen types shall be concentrated by using separation techniques. An example is given in Annex A for maize pollen shown with the matrix pollen load.

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

9.1 Microscopic pollen analysis

9.1.1 General

For any pollen type questioned in the GMO-monitoring, target parameters are primarily the collected pollen amount over the flowering period (all matrices), the daily rates (pollen loads) and the relative frequency of the pollen type to total pollen in the matrices.

9.1.2 Concentration in counts per gram of sample mass

From the pollen counts on the sample aliquot N_i on the mount (see 7.3) we calculate the pollen concentration of any target pollen type i in the sample matrix (honey, bee bread, pollen load) expressed in the unit pollen counts per g sample matrix according to Formula (2) by taking the ratio of the counted volume of the aliquot to the volume of the subsample into account and the respectively subsample mass:

$$C_i = \frac{\text{number of pollen counts}}{\text{aliquot volume}} \times \frac{\text{subsample volume}}{\text{subsample mass}} = \frac{N_i}{V_A} \cdot \frac{V_U}{M_U} [n \text{ pollen/g}] \tag{2}$$

where

C_i	is the number concentration of pollen type *i* in pollen counts per gram matrix;
N_i	is the number of pollen counts of type *i* on mount;
V_A	is the volume of counting aliquot *A* on the mount in µl;
V_U	is the volume of subsample *U* in µl;
M_U	is the mass of pollen in subsample *U* in g.

9.1.3 Total number of pollen collected per exposure time and season

The total number of collected pollen of type *i* over the exposure time respectively flowering season is estimated for any pollen type and matrix by multiplication of the concentration of the pollen type with the mass of the total sample of the matrix taking the respective flowering and sampling periods into account. In case of more than one sampling period the cumulated sum of pollen counts, pollen mass and sampling days are taken. In case of interval sampling of pollen loads, for example, the number of days sampled in relation to the whole sampling period of the bee colony respectively the whole flowering period related to the pollen type *i* shall be regarded:

$$N_{E,i} = \text{concentration} \times \text{sample mass} \times \frac{\text{total collection period}}{\text{sample days}} = C_i \cdot M_S \cdot \frac{T_P}{T_S} [n \text{ pollen}] \tag{3}$$

where

$N_{E,i}$	is the total number of collected pollen of type *i* per sample matrix and exposure period/season;
C_i	is the number concentration of pollen type *i* in counts per gram matrix;
M_S	is the sample mass in g;
T_S	is the number of days covered by the samples;
T_P	is the number of days for total collection period/flowering period of type *i* in the season.

9.1.4 Relative frequency

The relative frequency *H* of pollen type *i* in percent of the total pollen counts per sample is determined by for the whole sample over the exposure time and any single sampling period separately, too:

$$H_i = \frac{\text{number of pollen counts of type } i \times 100}{\text{number of total pollen counts}} = \frac{N_i}{N_T} \cdot 100[\%] \quad (4)$$

where

- H_i is the relative frequency H of pollen type i in percent of the total pollen counts per sample;
- N_i is the total number of collected pollen of type i in sample matrix and sampling period in counts per g sample matrix;
- N_T is the total number of all pollen types in counts per gram matrix.

9.2 Molecular-biological analysis

For the task of GMO-monitoring relevant to this Technical Specification, the results of the PCR shall be reported as percent GM related to the target pollen type (GM - DNA copies to pollen type DNA copies). The number of target pollen type counts per sample and per single PCR aliquot shall be reported, too.

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 positive;

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

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

10 Performance characteristics of the methods

10.1 General

The performance characteristics of the sampling method referring to the sampling procedure and microscopic analysis is described in detail in DIN 10760. It includes the accuracy and precision of the method according to ISO 5725-1[1)] [2] with detection limits, sensitivity and repeatability. The performance of the molecular-biological analysis shall be evaluated GMO-specifically (see the GMO-specific annexes).

10.2 Bee colony

Bee colonies are multifunctional, interactive biological organisms. Depending on their collecting activities, bee colonies represent the available pollen sources (→ foraging distance in 10.3). The bee colony can be partly standardized by good beekeeping practice [22; 33 and Annex C].

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

10.3 Foraging distance

Bee colonies, especially the foraging bees, actively roam an area and are therefore area related sampler.

Foraging distances and spatial distribution of foragers from one colony depend on spatial distribution of potential nectar, honeydew and pollen sources (melliferous and anemophilous plants), their actual honey and pollen flow as well as the requirements of the bee colonies. Whether plants are visited by bees depends on the attractiveness of the flowers as well as the preference of the bees. Honey and pollen flow depends also on weather and soil conditions. Requirements of the bee colonies depend on brood development and storage of feed.

The main foraging distances are [26]: modal distance from hive to forage site 0,7 km, median distance 1,6 km, mean distance 2,2 km, maximum > 10 km.

10.4 Honey samples, bee-bread samples and pollen load samples

Pollen in honey, bee-bread and pollen load samples reflects the spatial representation of melliferous plants for bees. Pollen from non-melliferous plants can also partly appear in these samples.

It is necessary to perform the sampling according to the standardized methods of this Technical Specification, including the documentation of sampling data.

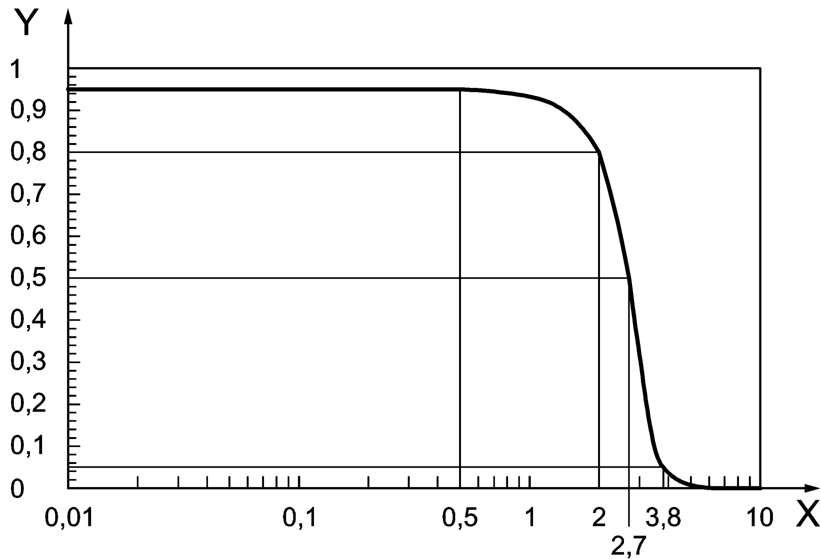
10.5 Microscopic pollen analysis

The performance characteristics for the microscopic pollen analyses is described in the validated standard DIN 10760.

10.6 Molecular-biological analysis

Regarding molecular-biological DNA-analysis per PCR the task of environmental GMO-monitoring differs to tasks of the food and feed sectors, which is reflected in the target parameters for GMO-monitoring (see Clause 9). The molecular-biological methods depend on the GMO and pollen type and have been evaluated for any GMO respectively pollen type specifically. Examples are given in Annexes A and B.

As an overall estimate for the performance of the method the probability for GMO-detection in a honey sample of a single bee colony in relation to distance to the GM-field as pollen source may be used (see Figure 1, example for GM-oilseed rape). The probability to detect GMO input is over 95 % up to a distance of approximately 500 m from the GM-field. The probability follows a sigmoid shape by increasing distance and is still 50 % at a distance of 2,7 km and drops below 5 % at distances greater than 3,8 km. This reflects the foraging distances in 10.3 in a plausible way.



Key

Y GMO detection probability

X distance in km

Figure 1 — Probability for GMO-detection depending on foraging distance respectively distance from GMO-source. Probability related to a honey sample of a single bee colony [14]

11 Quality assurance and quality control

11.1 General measurement strategy and task of pollen monitoring with biological samplers

The particular measurement strategy and conceptual task of pollen monitoring with biological samplers need to be specified.

11.2 Site protocol

The sites shall be documented and records shall be compiled which contain the following information:

— identification of the measurement site;

— exact position of the measurement shall be determined up to 10 m precision, plus elevation;

— the environment of the measurement site using topographic maps and possibly satellite images or aerial photographs from which the general landscape pattern can be derived: closer environment up to one kilometre and extended surroundings up to five kilometres;

— people responsible for the realization, contact person on site;

— date of installation of the measurement site;

— photographic documentation of the measurement site including the samplers.

If possible, all fields in the site's proximity in which plants of the same species as the target GMO are cultivated (e.g. rape, maize) shall be recorded on maps. Further the period of flowering should be stated.

11.3 Accompanying documentation for samples

The sample sheets shall contain:

— distinct and unambiguous identification of the sample results from the site, sample matrix, number of bee colony and date including calendar week of placement of the bee colonies;

— date of sampling;

— person taking the samples together with telephone number for consultation;

— weight of the sample;

— comments on noteworthy problems at the site, with the bee colonies or with the samples.

11.4 Parallel measurements

Parallel measurements using two colonies per site shall be carried out for at least 10 % of the sites.

11.5 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 the preparation of mounted reference samples for any relevant pollen type under identical 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.

Annex A
(normative)

Maize-specific requirements

A.1 Scope

This annex informs about maize specific requirements for GMO-pollen monitoring using the biological pollen sampling with bee colonies. 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 in some member states. Examples in the literature are shown by instance in [13] and [16].

The scope of this TS and annex is dedicated to environmental monitoring issues. This shall be distinguished from other purposes e.g. food and feed.

Main specific requirement: as matrix pollen loads shall be preferred.

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 [8; 12; 15]. 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 7 d to 14 d with the major portion of pollen being shed during midmorning to midday. In case pollen release is 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 wet weather 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 [17].

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: 65 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.

The flower produces no nectar, so that maize does not belong to primary host plants of bees. Maize flowers in the temperate regions of Europe, e.g. Germany, in summer. Despite of this, the pollen is rich of nutrients and being sampled by bees commonly in smaller quantities taken for nutritional purposes of the larvae. Therefore maize pollen are found regularly in the matrix pollen load. The average concentration of maize in pollen loads is between 0,1 % to 3 %, the daily values vary and may reach values higher than 50 % [13; 16]. In agricultural areas, maize pollen can be found in more than 50 % of summer honey but in smaller quantities around and below 1 %.

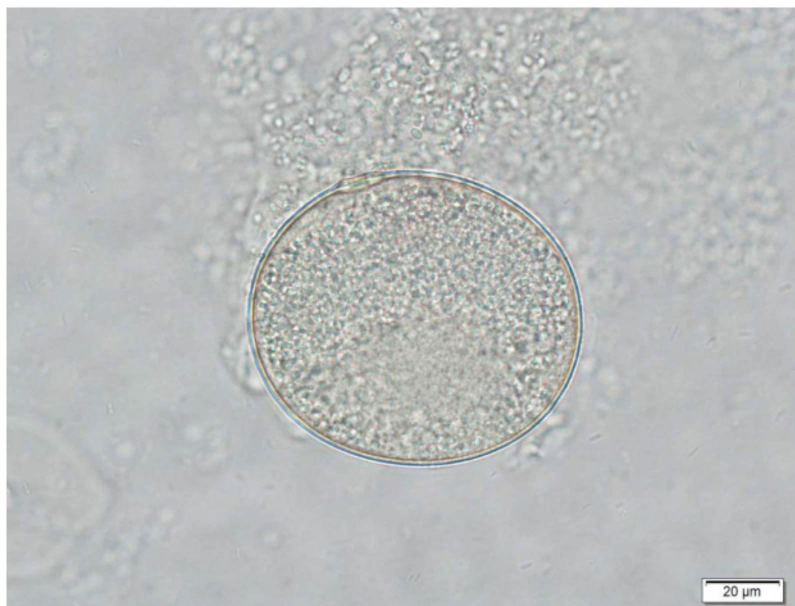


Figure A.1 — Maize pollen (*Zea mays*) under microscopic magnification [Source: Katharina von der Ohe, LAVES Institute for Apiculture Celle]

A.3 Sampling

In case of maize pollen, the sampling matrix shall be based on the pollen loads. The reasons are:

a) maize flowers in summer when most bee-keepers stop honey production;

b) the concentration and abundance of maize pollen in pollen loads is commonly higher enabling a better detection.

The sampling procedure follows the general description in Clause 5. For sampling the pollen loads traps in the bee hive are used to get a representative sample of the pollen loads collected by the bees. By using a modern trap type inside hive (see 5.2) an interval sampling of the two bee colonies shall be used with alternating sampling frequencies to cover the whole period (colony A day number 1, 3, 5…, colony B day number 2, 4, 6…).

The exact sample design depends on the particular task. In this annex an example is given. To ensure a valid detection of GMO by PCR, both the detection limit of the PCR and the respective required minimum number of maize pollen in the sample shall be regarded as the critical parameters.

For detection of GMO by PCR methods on a sensitivity level of 0,1 % GM (GM-maize/maize), a minimum of 10 000 maize pollen per sample as an empirical value should be aimed for [13].

A.4 Sample preparation

The sample preparation follows the steps described in Clause 6 for microscopic pollen analysis and Clause 7 for molecular biological analysis. This leads to two homogenized sample types: Half of the daily pollen load samples have been combined for a polled sample (A), the second half of the day samples are kept for further analysis (B series). For both types separation techniques shall be used to concentrate the maize pollen in the sample by separating the maize pollen from other pollen types and DNA-containing particles as far as possible. The method described here has been shown to avoid inhibition and enables a sensitivity on a 0,1 % level of GMO-detection [13]. The preparation shall be done uninterrupted to avoid still water conditions preventing germination of maize pollen and losses of DNA.

Maize pollen have a diameter range of 80 to 125 µm and therefore is larger than most other common pollen types. This enables a separation by filter sieving techniques (liquid sieving).

For detection of GMO by PCR methods on a sensitivity level of 0,1 % GM (GM-maize DNA copies / maize DNA copies), a minimum of 10 000 maize pollen per sample as an empirical value is necessary [13]. Therefore the amount of pollen load used for the molecular-biological analyses depends strictly on the results of the microscopic counting.

Necessary steps of procedure:

a) Depending on the results of the microscopic pollen counting (see 6.3), an equivalent amount of the homogenized sample of the total pollen loads (see sample B2, 8.2.2) is taken to achieve a minimum amount of 10 000 maize pollen, in the sample.

b) The homogenized pollen sample is further diluted with a tender detergent solution (0,01 % Tickopur[2]) in water,1:10) and thoroughly stirred for 5 min using a magnetic stirrer.

c) A vacuum cascade filter unit shall be applied with mesh sizes of 125 µm as pre-filter and of 63/64 µm for separation of the fraction including the maize pollen from smaller pollen and other particles like spores. The diameter and type of the sieve depends on the weight of the pollen load sample: For sample weights smaller than 10 g PA-filter ronds (50 mm diameter)[3]) (mounted on 1 000 µm PA-support filter ronds) (α) in a vacuum cascade filtration unit[4]) are preferably be used to minimize losses, thus the filter ronds with the maize pollen fraction can be transferred completely into 50 ml tubes for DNA-extraction. For sample weights greater than 10 g standard metal mesh sieves (test sieves) with 10 cm diameter (β) may be used (100 mm diameter, 40 mm height, mesh widths here 63 µm and 125 µm). For the stacked metal sieves a vibratory sieve shaker shall be used enabling a shaking frequency in the range of 100 Hz - 200 Hz and an amplitude in the range of 0,2 mm to 0,5 mm.

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2) 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) Filter ronds, polyamide (PA) sandwich meshes, diameter 50 mm, mesh sizes 64 µm (PA 64/45) and 125 µm (PA 125/45) on 1,000 µm support filter (PA 1,000/44). The second figure gives the percentage of open area. The filter ronds are supplied by TIEM technic GbR. 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.

4) 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.

d) The liquid together with suspended pollen (pollen suspension) is transferred into the vacuum filtration unit and the filtration is performed by adding Tickopur solution by thoroughly rinsing the meshes using a spraying nozzle (connected to the tap or a container under pressure) or alternatively a washing bottle.

e) The 63/64 µm filter retains the fraction of 63/64 µm to 125 µm with the maize pollen sample, which is used for the further analytical steps referring to the maize pollen monitoring.

f) After completion of the filtering the sample fraction containing the maize pollen will be transferred to 50 ml tubes for a second microscopic counting and DNA extraction.

g) -α- PA-filter ronds: The PA-filter ronds (diameter 50 mm) with the maize pollen fraction can directly be transferred to the 50 ml tubes. The pollen can now be washed off by rinsing and thoroughly shaking in the closed tube, than the filter is taken out. The supernatant liquid is removed by centrifugation (5 min, ca. 500 x g).

   -β- Metal mesh sieves with diameter 10 cm: The sample fraction with the pollen from the mesh is transferred to the 50 ml tubes by rinsing. The supernatant liquid is removed by centrifugation (5 min, ca. 500 x g).

h) The maize pollen samples are weighed inside the tube using the empty tube weight as tare.

i) The maize pollen sample 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 tubes with the maize pollen sample can be done frozen at −18°C or air-dried (for 48 h at room temperature).

A.5 Molecular-biological analysis of maize DNA using PCR

A.5.1 General

For detection of GMO the molecular-biological analysis of DNA using real-time PCR is described here. The method has been successfully developed and validated for maize [13; 16], the description here reflects the current state (February 2013). The procedure is done regularly first using the homogenized pooled sample for overall testing. The analysis is done at least with two parallel replicates. In case of results below detection limits, selected daily pollen load samples with higher maize content may be used for further analysis.

A.5.2 DNA extraction

a) Depending on sample size, an amount of 1-10 mg of the maize pollen sample is transferred in a second 50 ml centrifugal tube.

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. The maize pollen can now be counted in the suspension microscopically again.

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 5 mg to 1,000 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 (Picogreen dsDNA quantification assay).

A.5.3 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” [28] or in the respective web database for GMO methods. For the detection and quantification of GM pollen, methods should preferentially comply with the performance criteria set by the European Network of GMO Laboratories[5)].

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

All PCR on airborne samples shall 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 measuring 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.6 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 to total maize (GM maize DNA copies to maize DNA copies). 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 positive.

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

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

5) http://GMO-crl.jrc.ec.europa.eu/ENGL/ENGL.html.

Annex B
(normative)

Rapeseed specific requirements

B.1 Scope

This annex informs about rapeseed specific requirements for GMO-pollen monitoring using the biological pollen sampling with bee colonies. The molecular-biological detection of GMO is performed by analysis of pollen DNA using PCR methods.

This annex applies for all varieties of genetically modified (GM) oilseed rape varieties (*Brassica napus*) as well as conventional ones. It applies for pollen types with similar shapes of other Brassicaceae, too, by using the respective specific DNA-sequences for GMO-detection. The method described in this annex has been optimized for detection of genetically modified rapeseed pollen DNA by PCR for the task of a GMO-specific rapeseed pollen monitoring.

The scope of this TS and annex is dedicated to environmental monitoring issues. This shall be distinguished from other purposes e.g. food and feed.

Main specific requirement: As matrix honey shall be preferred.

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.

B.2 Basic principles

Rapeseed (*Brassica napus)*, also known as rape, oilseed rape, colza, canola, belongs to the Brassicaceae (= Cruciferae, mustard or cabbage family). Brassicaceae species are known for crossbreeding and rapeseed has a range of close related species and many varieties. Rapeseed is self-pollinated as well as insect pollinated (entomophil) and wind-pollinated (anemophil). The relative importance of self-, wind- and insect-pollination varies depending on local and weather conditions. The bright yellowish coloured flower produces nectar and is an attractive food source for honey bees and other insects [27].

Rapeseed pollen grains are of semi-angular shape (baseball-like) with varying diameters ranging from 20 µm to 30 µm and depending on the axial view, on average around 25-27 µm (see Figure B.1). The pollen is tricolpate and the exine has a light sticky surface, typical for entomophilous pollen. The pollen of most Brassica species are quite similar in shape and size and due to this difficult to distinguish microscopically, the usually achieved determination level is Brassicaceae [29].

The flower produces nectar and pollen, so that rapeseed belongs to the primary host plants of bees. Rapeseed is cultivated in the temperate regions of Europe, e.g. Germany, typically in two seasonal varieties, winter and summer rapeseed. Further, Brassicaceae are cultivated all over the year for other purposes, too. Rapeseed as well as other Brassicaceae seed spread easily and grow spontaneously at cultivated fields, gardens, roadsides and railways. Flower time extends from early spring to late fall or even early winter, until the first stronger frost period occurs. Cultivated winter rapeseed flowers typically over 3-5 weeks in April to May, depending on region (climate), weather, variety and cultivation conditions. The flowering period of cultivated summer rapeseed extends usually over a shorter period of 3-4 weeks depending on sowing time, cultivation and weather conditions from June to July.

Rapeseed pollen are found regularly and abundant in all pollen matrices in spring. Among the honey types, rape honey is a common honey type in spring. Typically pollen counts of rapeseed pollen in honey vary around several thousand to 15 000 pollen per g honey and the relative percentage of rape pollen to total pollen grains vary from a few percent up to more than 70 %. Rapeseed pollen can be found in summer honey, too, but usually in much smaller quantities.

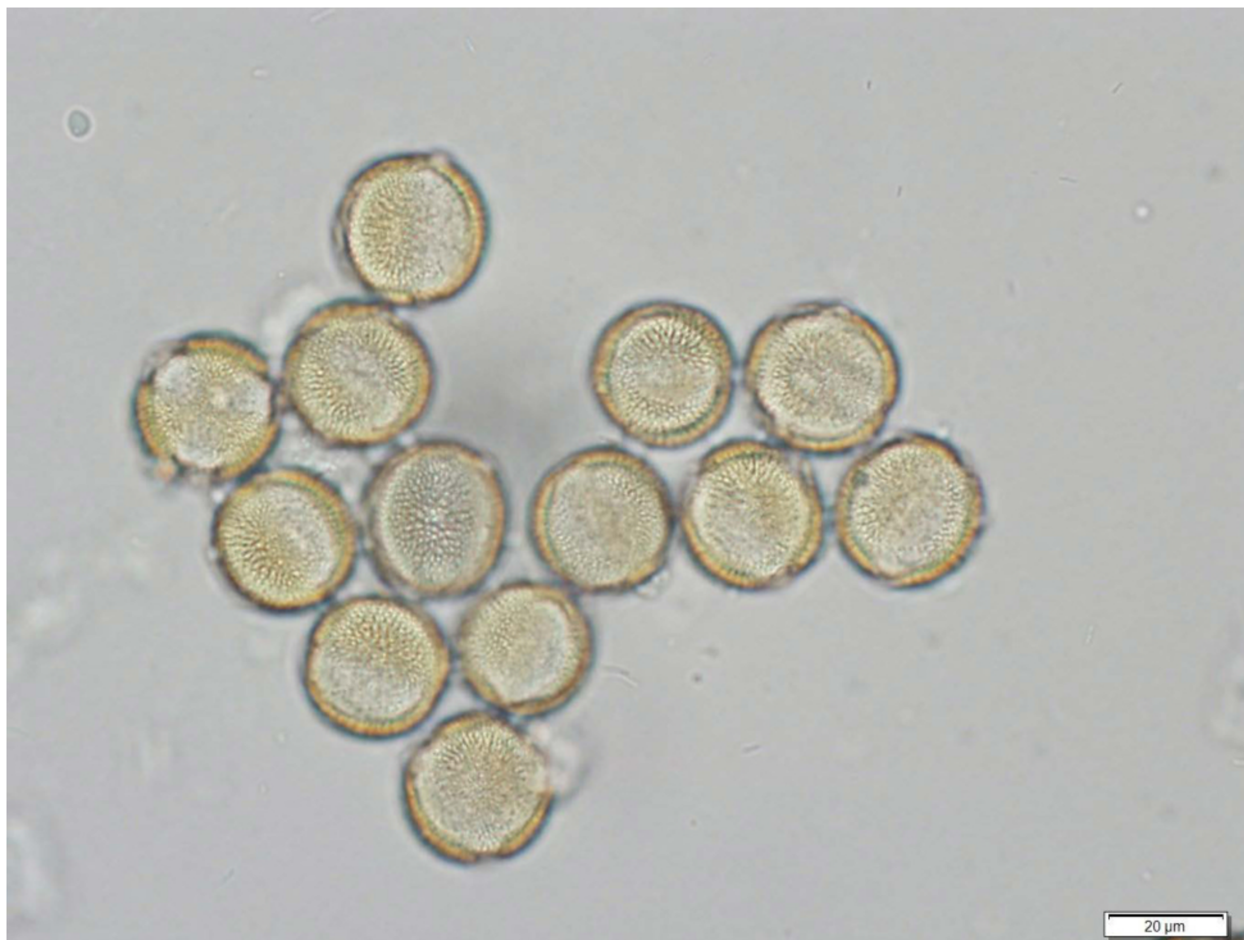

Figure B.1 — Rapeseed pollen (*Brassica napus*) under microscopic magnification [Source: Katharina von der Ohe, LAVES Institute for Apiculture Celle]

B.3 Sampling

In case of rapeseed pollen, the most advantageous sampling matrix to be preferred is honey. In case of special purposes only the matrix pollen load is more suitable, for instance if daily values are required or in summer. The reasons are: a) rapeseed is commonly and abundantly found in spring honey; b) honey is taken by the beekeeper so that no extra effort shall be undertaken for sampling; c) honey is the most homogenized matrix and gives one sample per period. This keeps the costs low for preparation and routine analysis. In this annex an example is given for the matrix honey. For using pollen loads, see Annex A.

The sampling procedure follows the general description in Clause 5. The matrix honey gives an integrated result over the sampling and respectively over the flowering period.

The exact sample design depends on the particular task. In this annex an example is given. To ensure a valid detection of GMO by PCR, the detection limit of the PCR shall be regarded and the respective required minimum number of rapeseed pollen in the sample as the critical parameter.

B.4 Sample preparation

The sample preparation follows the steps described in Clause 6 for microscopic pollen analysis and Clause 7 for molecular biological analysis. In case of a frequency of rapeseed pollen lower than 10 % in the sample, separation techniques shall be used to concentrate the rapeseed pollen by separating the rapeseed pollen from other pollen types and DNA-containing particles as far as possible. The method described here has been shown to avoid inhibition and enables a sensitivity on a 0,1 % level of GMO-detection [13]. The preparation shall be done uninterrupted to avoid still water conditions preventing germination of pollen and losses of DNA.

Rapeseed pollen have a diameter range from 20 µm to 30 µm. For separation of the rapeseed pollen fraction out of the honey matrix a liquid filter sieving techniques shall be applied using a vacuum filter cascade system with mesh sizes of 125 µm (pre-filter), 30 µm and 20 µm. The filter of mesh size 20 µm contains the up-concentrated rapeseed pollen fraction.

For detection of GMO by PCR methods on a sensitivity level of 0,1 % GM (GM-rape DNA copies/rape DNA copies), a minimum of 10 000 rapeseed pollen per sample as an empirical value is necessary [13] and for optimal performance an amount of 100 000 and more. Therefore the amount of honey used for the molecular-biological analyses depends strictly on the results of the microscopic counting.

Necessary steps of procedure:

a) Depending on the results of the microscopic pollen counting (see 6.3), an equivalent amount of the honey sample (see 8.2.1) is taken to achieve an optimal amount in the order of 100 000 rapeseed pollen and more, at least a minimum amount of 10 000 rapeseed pollen, in the sample.

b) The honey sample is further diluted with a tender detergent solution (0,01 % Tickopur[6)] in water,1:10) and thoroughly stirred for 5 min using a magnetic stirrer.

c) A vacuum cascade filter unit shall be applied with mesh sizes of 125 µm and 30 µm as pre-filters and 20 µm for separation of the fraction containing the rapeseed pollen from greater and smaller pollen and other particles like spores.

   (α) PA-filter ronds (50 mm diameter) (mounted on 1 000 µm PA-support filter ronds) in a vacuum cascade filtration unit[7)] are preferably be used to minimize losses, thus the filter ronds with the rapeseed pollen fraction can be transferred completely into 50 ml tubes for DNA-extraction.

   (β) For samples with a content of more than 10 g total pollen a cascade sieving using standard metal mesh sieves (test sieves) with 10 cm diameter may be applied before to minimize the sample (100 mm diameter, 40 mm height, same mesh widths). For the stacked metal sieves a vibratory sieve shaker shall be used enabling a shaking frequency in the range of 100 Hz - 200 Hz and an amplitude in the range of 0,2 mm to 0,5 mm. The outlet may be connected to a vacuum unit facilitating the filtration.

d) For performing the filtration, the diluted honey sample (b) with the suspended pollen (pollen suspension) is transferred into the vacuum cascade filtration unit (c) and the filtration is facilitated by thoroughly rinsing the meshes with the Tickopur solution using a spraying nozzle (connected to the tap or a container under pressure) or alternatively a washing bottle.

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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) 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.

e) The 20 µm filter retains the fraction from 20 µm to 30 µm with the rapeseed pollen sample, which is used for the further analytical steps referring to the rapeseed pollen monitoring.

f) After completion of the filtering the sample fraction containing the rapeseed pollen will be transferred to a 50 ml tube for a second microscopic counting and DNA extraction.

g) -α- PA-filter ronds: The PA-filter ronds (diameter 50 mm) with the rapeseed pollen fraction is transferred to a 50 mm petri disc for visual inspection and an optional second microscopic counting under the binocular or microscope (using the mesh grid as support for counting in lines). The filter rond with the rapeseed pollen sample is then transferred to a 50 ml tube ready for DNA extraction.

   -β- Metal mesh sieves with diameter 10 cm: The sample fraction with the pollen from the mesh 20 µm is transferred to the 50 ml tubes by rinsing. For a optional second counting, the volume is noted and 2 times of 10 µl are pipetted on a microscopic slide and counted later. The supernatant liquid is removed by centrifugation (5 min, ca. 500 x g).

h) The rapeseed pollen sample can be 1) analysed immediately or 2) stored for later analysis:

   1) For immediate analysis see B.6.

   2) Storage of the tubes with the rapeseed pollen sample can be done frozen at −18°C or air-dried (for 48 h at room temperature).

B.5 Molecular-biological analysis of rapeseed DNA using PCR for GMO detection

B.5.1 General

For detection of GMO the molecular-biological analysis of DNA using real-time PCR is described here [14; 13; 16], the description here reflects the current state (February 2013). The analysis is done at least with two parallel replicates.

B.5.2 DNA extraction

a) Depending on the sample size (number of the target pollen type in the sample), an amount of ca. 0,5 mg–5 mg of the pollen sample (minimum amount: at least 10 000 rapeseed pollen; rape pollen weight: ~10 ng) is transferred in a 50 ml centrifugal tube.

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.

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 rapeseed pollen (from 0,1 mg to 10 mg of rapeseed 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 fluorometric means (Picogreen dsDNA quantification assay).

B.5.3 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" [28] or in the respective web database for GMO methods. For the detection and quantification of GM pollen, methods should preferentially comply with the performance criteria set by the European Network of GMO Laboratories[8)].

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

All PCR on airborne samples shall 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 measuring 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).

B.6 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-rapeseed to total rapeseed (GM rapeseed DNA copies to rapeseed DNA copies). The number of rapeseed 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 positive.

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

— "GMO input cannot be detected [2-]" in case of consistent negative measurements.

8) http://GMO-crl.jrc.ec.europa.eu/ENGL/ENGL.html.

Annex C
(informative)

Good beekeeping practice

Approved beekeepers have the theoretical and practical tools to lead healthy bee colonies, efficient and appropriate for animals [11].

The beekeeper should:

— behave expertly at bee colonies;

— have a basic understanding of the biology of the honey bee;

— select suitable (e.g. good honey and pollen flow, appropriate microclimate, no disturbances by traffic) locations for bee colonies;

— evaluate properly the situation of honey flow and nutritional status of the bee colonies;

— have the ability to lead bee colonies through the entire year (e.g. overwintering, swarm control, harvesting honey, feeding, preparing for winter);

— assess development, strength, and health of bee colonies and perform appropriate and preventive treatment measures;

— successfully treat bee colonies against Varroose using an integrated operation plan;

— properly harvest honey according to legal guidelines;

— perform appropriate division of bee colonies;

— perform appropriate wax and comb hygiene;

— properly migrate bee colonies;

— properly clean and repair beekeeping equipment.

Bibliography

[1] CEN/TS 16817-1, *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*

[2] ISO 5725-1[9)], *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

[3] VDI 4330 *Part 1, Monitoring the ecological effects of genetically modified organisms — Genetically modified plants — Basic principles and strategies (Berlin: Beuth Verlag)*

[4] VDI 4330 *Part 3, Monitoring the effects of genetically modified organisms (GMO) — Pollen monitoring — Technical pollen sampling using pollen mass filter (PMF) and Sigma-2 sampler (Berlin: Beuth Verlag)*

[5] VDI 4330 *Part 4, Monitoring the effects of genetically modified organisms (GMO) — Pollen monitoring — Biological pollen sampling using bee colonies (Berlin: Beuth Verlag)*

[6] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC –Commission Declaration (OJ L 106, 17.4.2001, p. 1-39)

[7] 2002/811/EC: Council Decision of 3 October 2002 establishing guidance notes supplementing Annex VII to Directive 2001/18/EC of the European Parliament and of the Council on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC (OJ L 280, 18.10.2002, p. 27-36)

[8] AYLOR D.E., SCHULTES N.P., SHIELDS E.J. An aerobiological framework for assessing cross-pollination in maize. *Agric. For. Meteorol.* 2003, **119** pp. 111–129

[9] BATTESTI M.J., GOEURY C. Efficacité de l'analyse mélitopalynologique quantitative pour la certification des origines géographiques et botaniques des miels: le modèle des miels corses. *Rev. Palaeobot. Palynol.* 1992, **75** pp. 77–102

[10] BEHM F., VON DER OHE K., HENRICH W. Zuverlässigkeit der Pollenanalyse von Honig – Bestimmung der Pollenhäufigkeit. *Dtsch. Lebensmitt. Rundsch.* 1996, **92** pp. 183–187

[11] DADANT & SONS (1987): The hive and the honeybee, Hamilton

[12] EMBERLIN J., ADAMS-GROOM B., TIDMARSH J. (1999): A report on the dispersal of Maize pollen. Research paper. National Pollen Research Unit, University College Worcester, http://www.soilassociation.org

[13] FOLLONI S., KAGKLI D.M., RAJCEVIC B., GUIMARAES N.C.C., VAN DROOGENBROECK B., VALICENTE F.H. et al. Detection of airborne genetically modified maize pollen by real-time PCR. *Mol. Ecol. Resour.* 2012, **12** pp. 810–821

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

[14] HOFMANN F., SCHLECHTRIEMEN U., WOSNIOK W., FOTH M. (2005): GVO-Pollenmonitoring – Technische und biologische Pollenakkumulatoren und PCR-Screening für ein Monitoring von gentechnisch veränderten Organismen. BfN-Skripten 139, Bonn, http://www.bfn.de/fileadmin/MDB/documents/skript139.pdf

[15] HOFMANN F., JANICKE L., JANICKE U., WACHTER R., KUHN U. (2009): Modellrechnung zur Ausbreitung von Maispollen unter Worst-Case-Annahmen mit Vergleich von Freilandmessdaten. Federal Agency for Nature Conservation (BfN), Bonn, http://www.bfn.de/fileadmin/MDB/documents/service/Hofmann_et_al_2009_Maispollen_WorstCase_Modell.pdf

[16] HOFMANN F., EPP R., KALCHSCHMID A., KRUSE L., KUHN U., MAISCH B. et al. Schmidt. G., Schröder, W., von der Ohe, W., Vögel, R., Wedl, N., Wosniok, W. (2010): Monitoring of Bt-Maize pollen exposure in the vicinity of the nature reserve Ruhlsdorfer Bruch in northeast Germany 2007 to 2008. Umweltwissenschaften und Schadstoff-Forschung 22-3, p. 229–251, http://link.springer.com/article/10.1007/s12302-010-0133-6

[17] HOFMANN F., SCHLECHTRIEMEN U., KUHN U., WITTICH K.P., KOCH W., OBER S. et al. (2013): Variation of Maize Pollen Shedding in North Germany and its Relevance for GMO-Monitoring. Breckling, B. & Verhoeven, R. (eds.): Implications of GM-Crop Cultivation at Large Spatial Scales. Proceedings of the Third GMLS-Conference 2012 in Bremen. Theorie in der Ökologie 17, p. 19-25, http://www.gmls.eu/beitraege/19_Hofmann.pdf

[18] LANCASHIRE P.D., BLEIHOLDER H., LANGELÜDDECKE P., STAUSS R., VAN DEN BOOM T., WEBER E. WITZEN-BERGER, A., An uniformdecimal code for growth stages of crops and weeds. *Ann. Appl. Biol.* 1991, **119** pp. 561–601

[19] LOUVEAUX J., MAURIZIO A., VORWOHL G. Methods of Melissopalynology. *Bee World*. 1978, **59** pp. 139–157

[20] LUTIER P.M., VAISSIERE B.E. An improved method for pollen analysis of honey. *Rev. Palaeobot. Palynol.* 1993, **78** pp. 129–144

[21] PERSANO ODDO L., PIRO R., VON DER OHE W., VON DER OHE K. Main European unifloral honeys: descriptive sheets. *Apidologie (Celle)*. 2004, **35** pp. 38–81 [special issue]

[22] POHL F. *1 mal 1 des Imkerns. 1*. Auflage., Franckh-Kosmos-Verlag, Stuttgart, 2003

[23] RICCIARDELLI D'ALBORE G. *Textbook of melissopalynology*. Apimondia, Bucharest, 1997

[24] RICCIARDELLI D'ALBORE G. *Mediterranean melissopalynology*. Istituto di Entomologia Agraria, Università degli Studi, Perugia, 1998

[25] SAWYER R. *Honey identification*. Academic Press, Cardiff, 1988

[26] SEELEY T.D. *The wisdom of the hive: the social physiology of honey bee colonies*. Cambridge, 1995

[27] TREU R., EMBERLIN J. (2000): Pollen dispersal in the crops Maize (Zea mays), Oil seed rape (Brassica napus ssp. oleifera), Potatoes (Solanum tuberosum), Sugar beet (Beta vulgaris ssp. vulgaris) and Wheat (Triticum aestivum). Evidence from publications. A report for the Soil Association from the National Pollen Research Unit, University College Worcester, http://www.soilassociation.org

[28] VAN DEN EEDE G. (2001): Compendium of reference methods for GMO analyses, http://publications.jrc.ec.europa.eu/repository/handle/111111111/15068

[29] VON DER OHE K., VON DER OHE W. (2003): Celler Melissopalynologische Sammlung. Dt. Ausg., 2. Auflage, Hrsg.: Niedersächsisches Landesinstitut für Bienenkunde, Celle

[30] VON DER OHE K., VON DER OHE W. (2003): Celle's Melissopalynological Collection. Engl. Ed., 2nd edition, Hrsg.: Niedersächsisches Landesinstitut für Bienenkunde, Celle

[31] VON DER OHE W., PERSANO ODDO L., PIANA M.L., MORLOT M., MARTIN P. Harmonized methods of melissopalynology. *Apidologie (Celle)*. 2004, **35** pp. 18–25 [special issue]

[32] ZANDER E. (1951): Beiträge zur Herkunftsbestimmung bei Honig. Pollengestaltung und Herkunftsbestimmung bei Blütenhonig, Bd. I, Verlag der Reichsfachgruppe Imker, Berlin; Bd. II, Liedloff, Loth und Michaelis, Leipzig; Bd. III, Liedloff, Loth und Michaelis, Leipzig; Bd. IV, Ehrenwirth, München; Bd. V, Liedloff, Loth und Michaelis, Leipzig

[33] ZANDER E., BÖTTCHER F.K. *Haltung und Zucht der Bienen. 12. neubearbeitete Auflage*. Ulmer Verlag, Stuttgart, 1989

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