PD CEN/TS 16817-2:2015

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# 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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# Ambient air - Monitoring the effects of genetically modified organisms (GMO) - Pollen monitoring - Part 2: Biological pollen sampling using bee colonies

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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.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

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].

According to the CEN-CENELEC Internal Regulations, the national standards organizations of the following countries are bound to announce this Technical Specification: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.



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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].



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# 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



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**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

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**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.



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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).



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## 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).



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