2.11 Available methods for the sampling of nectar, pollen, and flowers of different plant species

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Abstract

Background: The new draft EFSA guidance document introduces additional assessment factors for pollinators other than honey bees. However, there are no standard test protocols available. Therefore, the only way for risk assessment refinements, are a more precise estimate of the potential exposure in nectar and pollen. The aim of the paper is to present available sampling methods of nectar and pollen but also tries to refine methodology for sampling of nectar and pollen mentioned in the guidance document.

Results: Nectar can be collected by hand from a wide variety of crop plants. This can be done with the help of capillaries as well as with centrifugation. Pollen can be collected with manual sampling or the help of a suction pump. Bees and bumble bees can be used for both matrices with many plants. Solitary bees are able to collect pollen. More detailed results are presented for oil seed rape and *Phacelia*.

Conclusion: Nectar and pollen can be collected from flowering crop plants visited by pollinators in amounts that are high enough to allow residue analysis. However, the minimum number of bees needed to collect the amount is not 20 but much higher, depending on the species of plant sampled. At least 200 honey bees should be collected for each matrix.

Introduction

The new draft EFSA guidance document on the risk assessment of plant protection products on pollinators[1] includes not only honeybees but also bumble bees and solitary bees. Additional assessment factors were introduced for bumble bees and solitary bees to account for their potential greater sensitivity, since there are no standard test protocols available for testing. For risk assessment refinements, a more precise estimate of the potential exposure via the expected residue values in nectar and pollen is possible.

The following sampling schedule and sampling amount is proposed in the EFSA draft guidance document:

Required are 5 trials per crop with immediate sampling after application, followed by 3 consecutive samplings. Possible sampling methods are manual sampling or sampling with the help of bees. For each sampling, 3 subsamples should be taken from at least 20 bees or plants. In order to obtain sufficient material for subsequent residue analysis, it is necessary to adapt the sampling methodology according to the specific morphology and the various pollen and nectar yields of the different plant species.

We will present our experience of nectar and pollen samplings with manual methods as well as with the use of honey, bumble and solitary bees for different plant species.

Materials and methods

Manual sampling methods

Nectar

One potential nectar sampling technique is the capillary method using micro-pipettes. Here, nectar will be sampled directly out of the flower with a micropipette collecting nectar with capillary forces in the tube (see Figure 1, Figure 2). This method is easy to use, but only possible in

species with sufficiently large nectar droplets. The micro-pipette sampling can be used, e.g. in cotton, citrus fruits, apple, tobacco, melon, and some oilseed rape varieties.

Figure 1 Sampling of nectar from oilseed rape *(Brassica napus L.*) flower with micro-pipettes.

Figure2: Nectar from apple *(Pyrus malus L.)* flower sampled with micro-pipettes.

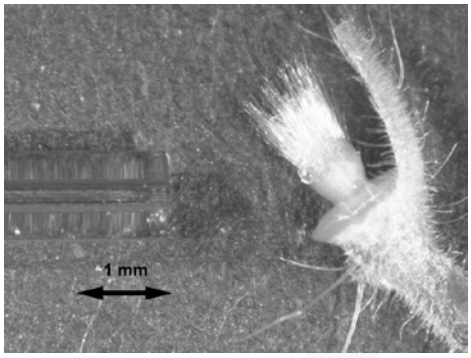

Figure 3 Nectar drop on Phacelia (*Phacelia tanacetifolia* Benth.) flower with micro-pipettes

Figure 4 Sampling of nectar from *Phacelia* flower with a centrifuge.

A relative new method developed for the sampling of nectar from flowers with small nectar droplets is centrifugation. This method was developed by Silva E.M., Dean B.B. and Hiller L. (2004) [2] for sampling of small flowers with less than 1 µL nectar. Flowers are collected in the field and if possible anthers are separated from the flower before centrifugation. An Eppendorf tube is prepared with an inlay filter (100 µm) to exclude plant parts from the nectar. The flowers will be put into the prepared tube with their opening facing the bottom of the tube (see Figure 4). The centrifuge will run for 2-3 seconds and the flowers will be replaced every time a new centrifugation starts. This will be repeated until the necessary amount of nectar is collected.

Pollen

A collection method for plants with a large number of flowers and heavier dry pollen is the beating of the flowers over a 500 µm sieve. Unwanted plant material can be taken out with a pair of forceps afterwards (see Figure 5). Crop plants where sieving is very succeful are oilseed rape and sunflower (*Helianthus annuus* L.). In some cases no free pollen is available and only anthers can be sampled. Anthers release the pollen from the inside after they are dried. Now the pollen can be sieved and the remaining material from the anthers removed. This will work for cotton (*Gossypium* sp. L) and apple. In wind fertilized species male flowers need to be enclosed with paper bags to collect the pollen in a sufficient amount. Two crop species where the method can be applied are maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* (L) Moench).

Figure 5 Sampling of pollen from oilseed rape flowers with a sieve.

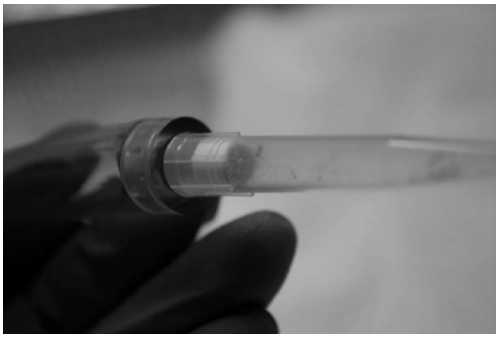

Figure 6 Sampling of pollen with a vacuum pump

One sampling method for sticky pollen is sucking the pollen out of the flower with the use of a vacuum pump. For this a pipette tip will be prepared with a filter and attached to the suction hose of the pump. The pollen from single flowers will be sucked into the pipette tip subsequently (see Figure 6). This will work well with species of the family *Cucurbitaceae*.

A further method to collect pollen from the family *Solanaceae* is sampling with an vibrating tool like an electric tooth brush, which works like visiting pollinators, increasing pollen release in some plants. Flower pollen can be collected by touching the flower with the vibrating tip. The pollen falling out of the flowers will be collected in a vial placed underneath the flower (see Figure 7).

Figure 7 Sampling of pollen from tomato with a modified tooth brush

Figure 8 Sampling of forager bees at the closed hive and directly from phacelia flowers.

Sampling with honey bees/ bumble bees

Sampling nectar and pollen with foraging bees

For samplings where honeybees (*Apis mellifera* L.) are used, only forager bees are collected. For the sampling, the hive entrance will be sealed and the forager bees will be collected either by

brushing them onto dry ice or by using a vacuum suction device (‘*bee vac*’) as they return to the hive (see Figure 8 and Figure 9). Alternatively, forager bees can also be sampled directly from the flowers. Afterwards the honey stomach will be prepared in the lab to obtain the nectar for the residue analysis (see Figure 10). This method can also be used for bumble bees (*Bombus* sp.).

Figure 9 Sample of forager bees with dry ice. Note the pollen hoses on the bees

Figure 10 Preparation of honey stomach from forager bees.

For pollen sampling, either pollen traps can be used or pollen can be collected from the prepared forager bees (see Figure 11). The efficacy of the pollen trap depends on the amount of pollen sampled by the bees. Some pollen is collected only in small amounts by the honeybees so the efficacy of the trap is limited. An efficacy of ≥ 50 % for all pollen sampled by the bees can be expected for a well fitting pollen trap. There are two basic designs available.The most commen design is a trap fitted in front of the hive before the hive entrance. An alternative design is only available for some hive measurements. There a drawer is slid between hive and the level where forage bees enter. The advantage of the design is the close fit.

Figure 11 Pollen trap for pollen sampling with drawer design

Figure 12 Sampling of stored oilseed rape pollen.

For bumble bees a special design for a pollen trap is needed since the workers size varies very widely. Here, brushes are used to remove the pollen hose from the bumble bees. At present, there is no efficacy known for this sampling method.

Sampling from the hive

A further method available is the direct collection of nectar and pollen from the hive of honey and bumble bees. For the sampling, empty cells are marked on the day before the sampling and sampled the following day. For pollen collection from honey bee combs a pollen lifter is a very useful tool.

Sampling with Red Mason Bees (*Osmia bicornis* L.)

Mason bees can only be used for sampling of pollen. For this method, nesting units will be placed in a tunnel within the crop. The pollen mass stored in the cavities by female *Osmia* will be sampled. One day before sampling the position of the last closed cell in each cavity will be marked with a permanent marker on the transparent cover of the assigned trays in order to sample the pollen mass from the desired date.

The pollen masses from at least 2 different cavities are usually sufficiently large to be analysed. The pollen mass is transferred by a spatula to sampling vials (see Figure 12).

Results and discussion

A detailed discussion for the two main bee food plants *Phacelia* and oilseed rape are given in the following text.

Table 1 shows all the samplings for different plant species performed by this working group over the last five years. The sampling with forager bees always included a set-up of a tunnel before sampling.

Table 1 Plant species where pollen or nectar has been sampled

Crops	Sampling by hand	Sampling with forager bees
Alfalfa (*Medicago sativa* L.)		x
Almonds (*Prunus dulcis* (Mill.) D.A.Webb)	x	
Apple (*Pyrus malus* L.)	x	x
Blueberry (*Vaccinium corymbosum* L.)		x
Buckwheat (*Fagopyrum esculentum* Moench)		x
Cherries (*Prunus avium* L.)	x	
Clover (*Trifolium repens* L.)		x
Coffee (*Coffea arabica* L. and *C. canephora* Pierre ex A. Froehner.)	x	
Cotton (*Gossypium hirsutum* L.)	x	
Elderberry (*Sambucus* sp. L.)	x (Pollen)	
Hemp (*Cannabis sativa* L.)	x (Pollen)	
Maize (*Zea mays* L.)	x (Pollen)	x
Melon (*Cucumis melo cantalupensis* L.)	x	x
Orange (*Citrus* × *sinensis* L.)	x	x
Oil seed rape (*Brassica napus* L.)	x	x
Olive (*Olea europaea* L.)	x (Pollen)	
Peach (*Prunus persica* L.)	x	
Phacelia (*P. tanacetifolia* Benth).	x	x
Potato (*Solanum tuberosum* L.)	x (Pollen)	x (BB)
Pumpkin (*Cucurbita sp.*)		x
Sunflower (*Helianthus annuus L.)*	x	x
Sorghum (*Sorghum bicolor* (L) Moench)	x (Pollen)	
Tomato (*Solanum lycopersicum* L.)	x (Pollen)	x (BB)
Vine (*Vitis vinifera* L.)	x (Pollen)	

BB - bumble bees used for collection, (Pollen) – only pollen can be sampled

Phacelia

Nectar

In *Phacelia tanacetifolia* it seems not possible to sample the necessary amount of nectar with micropipettes. In literature nectar amounts collected varied between 0.05 µl/flower up to 0.14 µl/flower[3]. Since the amounts are so small two options for the sampling of nectar are possible: sampling via centrifugation or sampling via forager bees. With boths methods samplings were performed succesfully in the past. Some further points which should be considered for the final choice of methods are:

- Number of samplings planned:
  The set-up of tunnels is work intensive for just one sampling and need more preparation time. On the other hand, sampling via centrifugation is manpower intensive on the sampling day
- For some active ingredients the residues may differ significantly according to the sampling method due to the contact of the applied material during sampling and the choice bees are making
- For very toxic compounds or compounds with a repellent effect on bees but not necessarily on other pollinators, a sampling with bees may not be possible directly after application

In the following, some data will be presented for nectar sampling with forager bees. A data set of 78750 bees was evaluated to estimate the amount of nectar collected. On average 227 of 1000 sampled forager bees contained measurable nectar amounts.

Table 2: Nectar content in sampled foraging bees in tunnels

Crop	Total number of sampled forager bees	Number of bees with nectar content in honey stomach	Percent of sampled forager bees containing nectar (%)	Total weight of nectar sample (g)	Mean amount of nectar for bees with nectar in stomach (g)	Necessary number of bees for a sample of 0.2 g
Phacelia	78750	21756	28	283	0.013	55
Oilseed rape	47409	7279	15	67	0.0092	145

The average amount of nectar obtained from one loaded stomach was 0.013 g. Based on this result, on average 55 forager bees with nectar are needed to get 0.2 g of nectar. This amount is usually needed as a minimum for subsequent residue analysis. Since the presence and amount of nectar in the honey stomachs is not predictable and since nectar amounts vary widely between samples from different varieties, field sites, weather conditions, and stages of flowering, a worker bee sample has to be much larger to get with a high certainty 0.2 g of nectar.

Pollen

A data set of 85161 forager bees sampled at the hive entrance was evaluated for the load of pollen. On average 229 of 1000 sampled forager bees carried pollen. From a subsample of 4972 forager bees 136 individuals with pollen load were taken and their pollen load was prepared and weighed, resulting in a total amount of 24 g of *phacelia* pollen.

Table 3 shows the results of this pollen amount evaluation. The average amount of pollen was 0.0048 g per individual. Based on this, 144 forager bees have to be sampled to get 0.2 g of pollen, which is often needed as a minimum amount for subsequent residue analysis. Since pollen load and the percentages of loaded bees varied widely between samples, attention has to be paid that for this purpose only forager bees with visible pollen load are sampled.

Table 3: Pollen load on sampled foraging bees in tunnels

Crop	Total number of sampled forager bees	Number of bees with pollen load	Percent of sampled forager bees with pollen load (%)	Total weight of pollen sample with forager bees (g)	Mean amount of pollen for bees with pollen load (g)	Necessary number of bees for a sample of 0.2 g
Phacelia	85161	24901 (4972)*	29	24*	0.0048	144
Oilseed rape	45171	27409 (7176)**	61	32**	0.0045	73

* only a subsample of 4972 forager bees with pollen load was prepared for the evaluation of the pollen amount

** only a subsample of 7176 forager bees with pollen load was prepared for the evaluation of the pollen amount

Oilseed rape

Nectar

Oilseed rape is known to be a good nectar source. In good conditions it can be sampled with a capillary. According to the literature [4], on average 2.33 µl/flower can be found with a variation between 1.1 up to 3.3 µl/flower According to our experience, for the sampling of 3 µL nectar by hand in a variety with good nectar production, about 6 flowers are needed. For 200 µL nectar about 400 flowers have to be sampled.

A data set of 47409 forager bees sampled at the hive entrance was evaluated for their nectar load. On average, 154 of 1000 sampled forager bees contained nectar in the stomach.

As Table 3 shows, the average amount of the loaded stomach was 0.0092 g. Based on this, an average of 145 forager bees is required to get 0.2 g of nectar. Since the presence and amounts of nectar in the honey stomachs are not predictable and since nectar amounts vary widely between samples from different varieties, field sites, weather conditions, and stages of flowering, a sample has to be much larger to get 0.2 g of nectar with a high certainty.

Pollen

A data set of 45171 forager bees sampled at the hive entrance was evaluated for pollen loads. On average, 607 of 1000 sampled forager bees carried pollen. From a subsample of 7176 forager bees, 155 individuals with pollen load were taken and the pollen load was prepared and weighed, resulting in a total amount of 32 g of *phacelia* pollen.

Table 2 shows the average amount of the pollen load was 0.0045 g. Based on this, 73 forager bees have to be sampled to get 0.2 g of nectar. Since pollen load and the percentages of loaded bees varied widely between different samples, attention has to be paid that for this purpose only forager bees with visible pollen load are sampled.

Conclusions

The results show clearly that it is possible to collect pollen and nectar from plants that are used in pollinator testing. Different sampling methods have been tried succesfully for the two main cultures, where manual sampling and sampling with pollinators can be used. The sampling with bumble bees and *Osmia* bees is an alternative to the sampling with honeybees that needs to be assessed further. It would be interesting to see if residues between the three species are comparable since it can be assumed that the foraging strategies are not always the same. Generally it has to be said that both methods, manually sampling and sampling with pollinators, are labour intensive. Detailed knowledge of plant physiology and ecology is needed to obtain sufficient sampling material. However, the 20 plants or bees given as a minimum requirement are only based on theoretical assumptions. To reach the amount of material needed for analytical analysis it is necessary to sample at least 200 honeybees for nectar and pollen each.

References

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