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Best Practice Documents for coexistence of genetically modified crops with conventional and organic farming

3. Coexistence of genetically modified maize and honey production

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3. Coexistence of genetically modified maize and honey production[†].

Ivelin Rizov and Emilio Rodrigues-Cerezo.

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[†] The mission of the JRC-IPTS is to provide customer-driven support to the EU policy-making process by developing science-based responses to policy challenges that have both a socio-economic as well as a scientific/technological dimension.

Joint Research Centre

This best practice document is the result of work carried out by the European Coexistence Bureau – Technical Working Group for Maize, consisting of the following European Commission staff and experts nominated by EU Member States:

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Executive summary

The Technical Working Group (TWG) for Maize of the European Coexistence Bureau (ECoB) analysed in 2010 the best practices for coexistence between GM maize crop production with non-GM maize[1]. In this document the analysis is extended to the coexistence between GM maize crop production and honey production in the EU. The TWG assessed if any further coexistence measure to those currently recommended in the previous document was required to limit adventitious presence of GM maize pollen in honey avoiding economic loses for producers. The terms of reference for this review are presented in Section 1. An overview of the structure of the honey-producing sector in Europe is given in Section 2.

The EcoB TWG maize held two meetings in June and November 2012 and examined state-of-art-knowledge from scientific literature, study reports and empirical evidence provided by numerous finished and ongoing studies looking at the factors determining the presence of pollen in general or maize pollen (even specifically GM maize pollen) in samples of EU produced honey. In addition to biological factors (related to honeybee behaviour and maize pollen characteristics) the TWG also analysed existing mandatory quality standards that impact the eventual presence of pollen in commercial honey. The review of this information (coming from a total of 136 references) is presented in a structured manner in Section 3 of this document. Finally, the TWG reviewed the state of the art and possibilities for the detection and identification of traces of GM maize pollen in honey (Section 4).

The analysis of existing information indicates that total pollen presence in honey ranges between 0.003 to 0.1 % in weight. Considering the share of maize pollen in total pollen found in honey, the extrapolated figures for maize pollen in honey would be around an order of magnitude lower. Nevertheless, it is important to stress that studies aiming at the detection/identification of this trace-levels of maize pollen are usually carried out with morphological identification and counting of pollen grains, and that a routine DNA analysis based on validated PCR protocol able to quantify total pollen in honey is unavailable. Once such a method could be found, the maize pollen fraction as well as the GM-pollen fraction of the total pollen could be established. In conclusion, the TWG maize of the ECoB, based on the analysis of the evidence summarised in this document concludes that no changes in the Best practice document on maize coexistence of July 2010[1] are necessary to ensure that adventitious presence of GM maize pollen in honey is far below legal labelling thresholds and even below 0.1 %.

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[1] Czarnak-Kłos, M, Rodriguez-Cerezo, E (2010) Best Practice Documents for coexistence of genetically modified crops with conventional and organic farming, Maize crop production, EUR 24509 EN

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1. Introduction

The foraging habits of honeybees are determined mainly by apiary size and the amount and variety of forage that a honeybee utilizes (Naug, 2009). Because landscapes in Europe have become increasingly characterized by intensively cultivated agricultural crops with a rotation of a few main species, and since honeybee pollination often occurs within a human-defined ecosystem, these crops could provide a significant part of honeybees' diet.

Almost all countries within the European Union grow maize. The cultivated area for maize production in the EU is about 13 million hectares. The area of grain maize production is about 8.4 million hectares, whereas for silage maize it is about 4.7 million hectares and for maize seed 95 thousand hectares are used. The total area for maize production comprises 13% of the cultivated area in the EU. The largest maize producers are France, Romania, Germany, Hungary and Italy, each growing more than 1 million hectares. Spain has about half of million hectares for grain and silage maize production. There is growing demand and support for EU maize production, due in part to its expanding use for ethanol and biogas production. Maize production in the EU is foreseen to further increase in the medium term and could reach about 70 million tonnes in 2020, establishing itself as the second most grown cereal after soft wheat, at the expense of barley.

Experience with commercial cultivation of GM maize in Europe is limited. In 2008, the cultivation of GM maize with the only authorised event, MON 810, was reported by 6 Member States (Czech Republic, Germany, Spain, Portugal, Romania and Slovakia) on an area of about 100,000 hectares (about 1.2% of the total EU maize acreage in 2008). In 2009, GM maize cultivation was discontinued in Germany and the total area planted in the EU decreased to about 95,000 hectares. Spain continues to be the largest EU grower of GM maize. In 2012 some 115,000 hectares were planted with Bt-maize in Spain, averaging 30% of the cultivated maize area in the country. However regional adoption varies considerably (ranging from 0% to over 80%).

The EU accounts for around 13% of global honey production, with 227,000 tonnes produced in 2009. Spain was the largest producer (33,000 tonnes), followed by Italy (23,000 tonnes), Hungary (22,000 tonnes), Romania (22,000 tonnes), France (20,000 tonnes) and Germany (18,000 tonnes).

Given the proposed further large scale extension of maize cultivation and widespread distribution of beekeepers in the EU (section 2: Structure and main products of apiculture in EU Member States), it is relevant to analyse the possible presence of genetically modified (GM) maize pollen in honey and other beehive products.

1.1. Legal Background

The European Commission proposed, on 21st September 2012, the amendment of Council Directive 2001/110/EC[1] to clarify the status of pollen in honey. In line with international FAO and WHO standards, the proposal defines pollen as a natural constituent of honey and not as an ingredient. The European Court of Justice (ECJ) ruling on Case C 442/09 (namely the Bablok case)[2] qualifies pollen as an ingredient in honey arguing that the pollen is found in honey mainly due to intervention by the beekeeper. However, pollen enters the hive as a result of the activity of the bees and is found in honey regardless of whether or not the beekeeper intervenes, therefore the Commission proposal recognizes that pollen is a natural constituent and not an ingredient of honey.

The Commission's proposal does not affect the conclusion of the ECJ as regards the application of the GMO legislation to GM pollen in food. In particular honey containing GM pollen can be placed on the market only if it is covered by an authorisation under Regulation (EC) No 1829/2003[3] on GM food and feed. Furthermore, the GM labelling rules referred to in Article 12 of Regulation (EC) No 1829/2003 and in Article 4 of Regulation (EC) No 1830/2003[4] are applicable. The relevant labelling threshold of 0.9% of the total product, according Article 12(2) of Regulation (EC) 1829/2003, should be considered.

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[1] Council Directive 2001/110/EC of 20 December 2001 relating to honey. OJ L 10, 12.1.2001, p. 47.

[2] OJ C 24, 30.1.2010, p. 28 and OJ C 311, 22.10.2011, p. 7.

[3] Regulation (EC) No 1829/2003 of the European parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003, p.1.

[4] Regulation (EC) No 1830/2003 of the European parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC. OJ L 268, 18.10.2003, p.24.

Due to the possible interaction between the different production lines in agriculture, as an open system, their coexistence determines freedom of customer's choice through the food chain. In that respect adequate technical and organizational measure may need adoption, according Article 26a of Directive 2001/18/EC[5] between genetically modified (GM) maize and honey production. Application and efficiency of these coexistence measures are closely linked to the local conditions such as climate and farm structure conditions. Therefore Member States have the flexibility in definition and adoption of such measures, according Commission Recommendation on development of national co-existence measures to avoid the unintended presence of GMOs in conventional and organic crops from 13 July 2010[6].

The organic production of honey are regulated by the Commission Regulation (EC) No 889/2008[7], defining the rules for implementation of Council Regulation (EC) No 834/2007[8] on organic production and labelling of organic products, with regard to the production conditions, labelling and control. According to article 13 of this regulation, apiaries shall be placed in a way that within a radius of 3 km nectar and pollen sources consist essentially of organically produced crops and/or spontaneous vegetation and/or crops treated with low environmental impact methods. Furthermore for inspection purposes, control bodies of the Member States have to receive a map on an appropriate scale from beekeepers listing the location of the hives and the area where the apiary is placed shall be registered together with the identification of the hives (Article 78 of the Commission Regulation (EC) No 889/2008).

1.2. The role of the European Coexistence Bureau

The European Coexistence Bureau (ECoB), Technical Working Group for maize (TWG maize) was asked to discuss if the current TWG maize recommendations highlighted in the Best Practice Document (BPD) on maize coexistence of July 2010 (Czarnak-Kłos M, Rodriguez-Cerezo E, 2010) address sufficiently the issue of coexistence of GM maize and honey production in the context of the proposed legislative change.

If not sufficient, the TWG maize was asked to propose, based on current scientific knowledge and agricultural practices, additional coexistence measures to limit GM maize pollen presence in honey to the required levels that would impose the minimum cost and burden for both farmers and beekeepers.

1.3. Scope of BPD document

The Best Practice Document will cover only coexistence between EU GM maize crop and honey production, with reference to methods for quantification of GM pollen in honey.

The coexistence measures should be addressed to GM maize producers. Measures could also be advised for beekeepers as well in order to assure coexistence in both production streams. All these measures should be proportional, technically and economically consistent.

The thresholds for coexistence to be analysed are the legal labelling threshold (of 0.9%) and the limit of quantification (of about 0.1%), which is commonly required by operators in some markets. These two different coexistence thresholds are in line with the Commission Recommendation of 13 July 2010[6].

The review considers GM maize with a single transformation event and the foraging behaviour of honeybees (*Apis mellifera L.).*

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[5] 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. *OJ L 268, 18.10.2003, p.21.*

[6] Commission recommendation of 13 July 2010 on guidelines for the development of national co-existence measures to avoid the unintended presence of GMOs in conventional and organic crops. *OJ C 200,* 22.7.2010, *p.1.*

[7] Commission regulation (EC) No 889/2008 of 5 September 2008 laying down detailed rules for the implementation of Council Regulation (EC) No 834/2007 on organic production and labelling of organic products with regard to organic production, labelling and control. OJ L 250, 18.9.2008, p.1.

[8] Council Regulation (EC) No 834/2007 of 28 June 2007 on organic production and labelling of organic products and repealing Regulation (EEC) No 2092/91. *OJ L 189,* 20.7.2007, *p.1.*

2. Structure and main products of apiculture in EU Member States

The major producers of honey in the EU are: Spain, Germany, Romania, Hungary, France, Greece, Poland, Bulgaria and Italy (FAOSTAT, 2010). Each of them counts more than 100,000 beehives. In most of these countries, as: Spain, Romania, Hungary, France, Greece and Bulgaria as well as in Portugal, Netherlands and Lithuania apiculture is experiencing a trend towards enlargement in the size of production units (i.e. number of hives) whilst overall the number of apiaries continues to decline (Rodrigo, 2011 and table 1).Beekeepers are classified as professionals, semi-professionals or amateurs. Categorization as professional or amateur is based on income and/or the number of beehives. Annex II of Regulation (EC) 917/2004[9] defines a professional beekeeper as anyone operating more than 150 hives.

According to the Commission report of 2003 to the Council and the European Parliament on the application of Regulation (EC) No 1221/97[10], professional beekeepers exploit 43.7% of European beehives. Spain had the highest rate with 74% of beehives managed by professional beekeepers, followed by Greece and Portugal with more than 50% and France with 45%. The rates of professionalism for year 2010 were: for Spain - 80.5%, for Greece - 62.7%, for Portugal - 40.4% (Rodrigo, 2011) and for France - 54.4% (FranceAgriMer, 2012). Despite a steady decline in the number of farms practising beekeeping, the average number of hives in production per farm has steadily increased or stabilized at achieved level (FAOSTAT, 2010).

Table 1 Structure of apiculture in some EU Member States*

Country	Beekeepers			
	Total number	Professional, %	Semi-professional, %	Amateur, %
Austria	24,450	1.0	-	99,0
Bulgaria	29,244	1.1	11.5	87.4
Denmark	-	2.0**	-	98.0
Germany	80,400	0.5	-	99.5
France	41,836	3.9	6.9	89.2
Ireland	-	1.0**	-	99
Lithuania	-	2.5**	-	97.5
Netherlands	8,000	2.5**	-	97.5
Poland	44,951	0.5	9.5	90.0
Romania	5,432	19.5	23.9	56.6
Spain***	24,230	19.5	-	80.5
Slovakia	16,239	1.1**	-	98.9

* *data are reported by members of the TWG for maize of ECoB or from open literature sources*
** *with over 100 hives*
*** *dated April 2012 (Honey sector in figures, May 2012)*

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[9] Commission Regulation (EC) No 917/2004 of 29 April 2004 on detailed rules to implement Council Regulation (EC) No 797/2004 on actions in the field of beekeeping. *OJ L 163, 30.4.2004, p.86.*

[10] Council Regulation (EC) No 1221/97 of 25 June 1997 laying down general rules for the application of measures to improve the production and marketing of honey. *OJ L 173, 01.07.1997, p.1*

In 2010, seven out of ten apiaries had less than 30 hives, and these were responsible for only 7% of France's annual honey production (Lerbourg, 2012). Two-thirds of farms with beekeeping represent economically weak, small farms, all managing less than 150 hives. In 2010, 6% of beekeepers in France had 63% of the hives and delivered 72% of the apicultural production (FranceAgriMer, 2012). This trend towards production concentration is also common in other Member States. EU apiculture is becoming more professional with a decline in amateur beekeepers (less than 30 hives) and the stabilization of the group of professional beekeepers who strengthen their relative weight in terms of the number of hives.

Small scale operators, mainly amateur beekeepers, supply beehive products for their own consumption or local outlets. In this case most products are sold directly by the beekeeper to the final consumer. Direct sales to the final consumer for 2010 in Bulgaria experienced a 6.4% downturn and accounted for 30.1% of the total marketed honey in this country (Agri Report, 2011). Diversification of markets - wholesale, semi-wholesale and direct sale - may appear a secure option, but the costs and the general overtime related to marketing, plus the difficulty of building up a loyal clientele, cannot, in most cases, be afforded by small farms producing as amateur and semi-professional beekeepers.

The sociological status of beekeepers on EU farms in terms of human labour units is categorized as a relatively small scale personal operation. Two categories are clearly distinguishable: active farmers (handling more than 70 hives) and retired people (usually with less than 70 hives) (FranceAgriMer, 2012; Semkiw and Skubida, 2010). Most of these beekeepers also have another professional activity. The retirees also comprise a significant number of the beekeepers in other EU countries such as Austria, Czech Republic, Slovakia, Ireland, and the Netherlands.

The turnover of the beekeepers in all EU countries depends essentially on the honey production, which is the significantly predominant beehive product. Over 75% of farms surveyed in France (FranceAgriMer, 2012) indicate that honey is responsible for more than 85% of their turnover. Amateur beekeepers with less than 10 hives focused solely on honey production. The economic value of other beehive products averaged 1.3% for pollen, 0.3% for propolis, 2.7% for royal jelly, and 0.2% for beeswax production. In addition to beehive product supply, there are swarms, queens and livestock productions.

Honeybees are now managed not only to produce honey but also to serve as pollinators of many cultivated plants, although maize is not one of them. The provision of honeybees for the pollination of crops is a specialized practice, not just a sideline of honey production. This activity is carried out mainly by professional beekeepers. The currently ongoing FP7 research project STEP (with duration from 01/02/2010 to 31/01/2015) aims to document recent statuses and trends in pollinators and insect-pollinated plants in the EU. It will take major strides towards filling current knowledge gaps regarding pollinators.

EU apiculture produces mainly poly-floral honey. In addition to it rapeseed and sunflower unifloral honeys represent significant volumes but their value is comparatively low. Orientation of production towards high-valued unifloral honeys results in better recovery of the production costs.

The main unifloral honey produced in the EU is acacia honey, as the black locust tree from which it is obtained is widely spread in Europe. The main producers of acacia honey in Europe are Hungary, Bulgaria and Romania, although it is also produced in other EU countries. Other types of unifloral honey commonly produced in the EU are: rapeseed, sunflower, linden blossom, heather, lavender, rosemary, thyme, orange blossom, chestnut and forest honey.

The average yield per hive for professional beekeepers in France for 2004 ranged from 12 kg per hive to 56 kg per hive, with an estimated average national production of 24 kg per hive. For beekeepers with less than 150 hives, an average production of 18 kg per hive was reported, with values ranging from 8 kg per hive to 40 kg per hive (Gem-Oniflhor, 2005). There is a clear positive relation between the number of hives managed and the average yield obtained per hive.

Extracted honey is the most basic and widespread hive product. It is obtained by centrifuging decapped broodless combs. For example, in Ireland it comprises 97% of marketed honey (in a communication with John Claffey). In addition to honey obtained by centrifugation, in the EU market there are niche products such as comb honey and pressed honey, however only limited data on their market share are available. It is estimated that in Ireland comb and pressed honey comprise 2% and 1% of marketed honey respectively. Pressed honey production is a very local activity, usually in regions outside of intensive agricultural activities.

3. Review of available information on appearance and management of adventitious presence of GM maize pollen in honey

3.1. Honeybees foraging

Honeybees can forage for conventional maize pollen as well as for GM Bt-maize pollen (Lipiński et al., 2008, Malone and Pham-Delegue, 2001). Therefore, studies on honeybees foraging for maize pollen also have to be considered for examination of the possible introduction of GM maize pollen in beehive products.

3.1.1. Studies on ranges of flight distances

In agricultural areas honeybees commonly forage for water, pollen and nectar in a distance range of several hundred metres from their hive (Free, 1970; Michener, 1974; Beekman et al., 2004). The foraging distances depend on:

- Abundance, variety and size of profitable forage sites and landscape structure (Seeley, 1987; Waddington et al., 1994 ; Beekman and Ratnieks ,2000; Beekman et al. 2004; Visscher and Seeley, 1982; Steffan-Dewenter and Kuhn, 2003);
- Size and developmental stage of the colony (Visscher and Seeley, 1982; Schneider and McNally, 1993; Schneider and McNally, 1993; Schneider and Hall, 1997; Beekman et al., 2004);
- The heritable behaviour of pollen and nectar collection. European colonies can be selected for high and low pollen collection behaviour (Hellmich et al., 1985; Calderone and Page, 1988, 1992; Page and Fondrk, 1995), and there can be subfamily differences within colonies for pollen versus nectar foraging (Robinson and Page, 1989; Robinson, 1992; Guzman-Nova et al., 1994). Subfamilies within colonies can exhibit genetically determined differences in foraging distance preferences and in the plant species visited for pollen (Oldroyd et al., 1992, 1993).

In table 2 the mean flight distances covered by forage honeybees are listed. All of them are revealed by decoding of the dance language of honeybees by which they communicate the distance and location of food resources.

Table 2 Mean foraging distances of honeybees estimated by decoding their dance language

Number of studied colonies	Location and plant environment	Mean forage distance	Reference
1	New York, USA (Temperate deciduous forest)	**666 m** - **2031 m** (total foraging)	Visscher and Seeley, 1982
2	New York, USA (Buckwheat patches in a forested environment, poor in forage)	**in 1000 m** (70% of the colonies discovered them), **in 1900 – 2000 m** (50% of the colonies discovered them), **in 3200 – 3600 m** (0% of the colonies discovered them)	Seeley, 1987
4	Suburban environment in: Florida, USA (FL1 and FL2 colonies)	**707 m** (pollen foraging) **803 m** (nectar foraging) **821 m** - **664 m** (colony FL1 and FL2 variation)	Waddington et al., 1994
	California, USA (CA1 and CA2 colonies)	**705 m** (pollen foraging) **899 m** (nectar foraging) **1138 m** – **534 m** (colony CA1 and CA2 variation)	
3[1]	Guanacaste , North-western Costa Rica	**1387 ± 260 m** (total foraging) **1402 ± 336 m** (pollen foraging) **1202 ± 82 m** (nectar foraging)	Schneider and Hall, 1997

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[1] European-African honeybee hybrid.

Number of studied colonies	Location and plant environment	Mean forage distance	Reference
4	Southern Lower Saxony, Germany:		Steffan-Dewenter and Kuhn, 2003
	2 structurally simple landscapes locations	**1569 ± 55.6 m** (total foraging) **1743.4 ± 96.6 m** (pollen foraging)	
	2 structurally complex landscapes locations	**1488.9 ± 49.9 m** (total foraging) **1543.4 ± 70.97 m** (pollen foraging)	
		1526 ± 55.6 m (total foraging, an average among colonies and locations)	
		time (resources availability) variation **1319 ± 53.2 m** (total foraging in May, abundance of resources) **1786.9 ± 96.6 m** (total foraging in June, scarce of resources) **1518.2 ± 51.3 m** (total foraging in July, moderate resource availability)	
2 small colonies with 6000 bees 2 large colonies with: 21000 and 18000 bees	Sheffield, Yorkshire, UK	**670 m** (abundant forage - July, small colonies) **620 m** (abundant forage - July, large colonies) **1430 m** (scarce forage - August, small colonies) **2850 m** (scarce forage - August, large colonies)	Beekman et al., 2004
2 different colonies with ≈ 4000 workers honeybees, because the first did not survive winter	Sheffield , Yorkshire, UK (extensive patches of heather were in bloom on moors in the Peak District west of Sheffield)	**1000 m** (May, before heather blooming) **5500 m** (August, blooming period of heather)	Beekman and Ratnieks, 2000

Three of the studies listed in table 2 (Waddington et al., 1994, Schneider and Hall, 1997 and Steffan-Dewenter and Kuhn, 2003) present data on the mean distances flown by worker honeybees for pollen foraging. Although their experimental design taking into account differently the factors affecting the flying behaviour of honeybees, such as environment, vegetation and landscape, and heritable colony characteristics, one rough estimation of the mean flying distance for pollen foraging is averaged of about 1200 m.

Other factors that influence honeybees' flying range as availability of foraging resources and size of colonies also should be considered for averaging of flying distance for pollen foraging. From the works of Steffan-Dewenter and Kuhn, 2003 and Beekman et al., 2004 (table 2) can be estimated a 136% increase of foraging distance, as the correction coefficient in the scarce of forage.

It should be pointed out that the revealed estimation of the mean pollen foraging distances of honeybees is only an initial step for its determination, which requires additional research.

The energy consumption of a flying honeybee is about 0.5 mg honey per kilometre. In order to provide one kilogram of surplus honey for market the colony has had to consume something like a further 8 kg to keep itself going (Crane, 1975). Therefore the maximum foraging ranges for honeybees of up to 13500 m and 9500 m reported by Von Frisch (1967) and Beekman and Ratnieks (2000) should only be attributed to scout honeybees searching for feed resources (Beekman et al., 2007) or to a starving colony's attempt to survive in a landscape with scarce resources, and should not be interpreted as common behaviour of forager honeybees.

Another reason for long flight distances of honeybees could be the purpose of exploitation of highly rewarding and attractive patches of vegetation such as heather (*Calluna vulgaris*) (Beekman and Ratnieks, 2000), which is one of the main sources of nectar across the EU (Crane et al., 1984). Honeybees select forage plants primarily on the basis of the sugar content of the plant nectar or the honeydew, the raw material of honey (Crane, 1980; Seeley, 1995).

In addition to the high energy consumption during foraging over long distances, the natural process of pollen exchange caused by the honeybee should be considered (Crane, 1980). During the return flight pollen could become loose due to weather conditions (Seeley, 1995).

After Von Frisch's (1967) discovery that worker honeybees communicate with nestmates via the round, sickle and waggle dances, researchers have studied many aspects of the dance language: mechanisms and evolution of message production; message reception; the role of odour, memory, and acoustics; and how honeybees measure distance. Even these achievements, the quantification and decoding of waggle dances, present certain experimental challenges (Couvillon et al., 2012).

The findings of Srinivasan et al. (2000) show that honeybees measure distances by optic image flow and not by energy consumption and that communicated distances may depend on the nature of the landscape through which the bee flies (Esch et al., 2001). This could result in a systematic error, i.e. honeybee dances in landscapes with low optic flow. Therefore Steffan-Dewenter and Kuhn (2003) concluded that the reported differences in foraging distances covered by honeybees in simple and complex landscapes may have been an artefact. The main benefit of the honeybee's dance communication seems to be that it enables the colony to forage at the most profitable patches only, ignoring forage patches that are of low quality (Beekman and Lew, 2008).

Even though the use of digital video and computer techniques makes it possible to review footage easily, allowing for after-the-fact dance decoding, the decoding of simultaneous dances and more accurate measurement of orientation, dance decoding remains time-consuming (e.g. a single forager bee may make waggle runs for over an hour in real time). Therefore, there is a need for protocols to optimise dance decoding (Couvillon et al., 2012).

All these uncertainties regarding the determination of forage distances by decoding the dance language of honeybees are overcome in the work of Hagler et al. (2011). The authors introduced a non-intrusive marking method for tracking the natural behaviour of insects. They examined the foraging range of honeybees in an alfalfa seed producing field, located in an intensively managed agricultural area. Self-marking devices were placed on 112 selected honeybee colonies originating from nine different apiary locations. The hives in each apiary contained a distinct mark, which enabled identification of the apiary of origin and distance travelled by each marked field-collected honeybee. Over two years a grand total of 12266 bees (4391 for the first and 7875 for the second) were collected. The study revealed that the number of forager honeybees decreases exponentially with distance. On average, honeybees travelled 738 m and 865 m from their apiary in the first and second years respectively. However, the flying distances of marked honeybees ranged from a minimum of 45 m to a maximum of 5983 m.

The exponential decay of number of forager honeybees within flying distances, and the average distance travelled (around 800 m) identified with this experimental approach correlates with findings obtained by the decoding of honeybees' waggle dance (table 2: Visscher and Seeley, 1982; Seeley, 1987; Waddington et al., 1994; Beekman et al. 2004). The conclusion is that the honeybee colonies can monitor a large area, exploiting a large number of sites, but are focused on only a limited number of patches, most likely to be the most bountiful near the hive.

The presented estimation of about 1200 m for the mean distance of honeybees' pollen foraging, under normal conditions, is roughly in line with the conclusion that common forage distances vary from few hundred to a thousand meters. The validity of this conclusion is reinforced when the naturally occurring, stochastic distribution of worker honeybees within the flying distances is taken into consideration (Beekman and Ratnieks, 2000).

None of the above presented studies that assess the foraging range of honeybees provide information to infer the flying distances covered for effective maize pollen transfer to the hive and into honey. However, as concluded here, it is unlikely that worker honeybees will forage maize pollen beyond distances of a few hundred to a thousand metres. This assumption is backed up by the fact that maize is not a nectar producing species, which means that the energy consumed by flying to maize plants, is derived from resources already stored in the hive or the worker honeybees must previously visit other plants for nectar collection. During these visits honeybees may also collect pollen and will not necessarily visit maize plants for further pollen foraging (especially when it is not among the most attractive and profitable pollen sources, section 3.1.3).

This conclusion from the analysis of flying distances covered by honeybees foraging for maize pollen, of about a thousand metres, is complemented well by the works of Hofmann et al., 2010 and Rosenkranz, 2008 (section 3.1.4.). Hofmann et al. (2010) found a decrease in the Bt-maize pollen content in the total harvested pollen of about 93% by increasing the distances (with 150 m in a northerly and 400 m in a westerly direction) between beehive and maize fields. Rosenkranz (2008) monitored the foraging of eight honeybee colonies placed up to 1 km from maize fields in Baden-Württemberg and also reported that the amount of maize pollen which entered the beehive decrease with an increase in distance from the maize field and in a distance of 1 km GM maize pollen is only detectable by PCR, which means that its content is about or below of 0.1% w/w, according to the limits of detection and quantification for the maize event MON810 (ISO/FDIS 21570:2005).

The legally established distance requirements for organic production of honey (article 13 of Commission Regulation (EC) No 889/2008) that apiaries can only be placed in areas with nectar and pollen sources consisting essentially of organically produced crops within a radius of 3 km, is about three times bigger than the roughly estimated flying distances covered by honeybees for maize pollen foraging under normal condition. The practical value of such a comparison must be confirmed by further research due to the limited data available presently and the large number of factors influencing the flying distance of forager honeybees. However, it is clear that for the quantification of GM maize pollen in honey at bigger distances from maize fields, e.g. 3 km, the currently available standardized analytical procedures must be adjusted accordingly, since the investigated quantities most likely will be far bellow their detection limit of ≤0.1% w/w (section 3.2. and 4), as is already reported by Mildner et al. (2011).

3.1.2. Maize pollen grain features

Maize produces pollen over a 14-day period (Paliwal, 2000; Sleper and Poehlman, 2006). Pollen is shed continuously for a week or more from each plant, starting approximately 1 to 3 days before silk emergence. Maize pollen is naturally designed for wind dispersal as the maize plant is non-melliferous and congenitally has a smooth spherical shape.

The size and the weight of maize pollen grains are naturally varied. The factors that influence the physical dimensions of pollen grains are their origin and climate conditions (temperature and humidity) during development (Blance, 1950). In addition, a significant biological variation among individual plants remains (Kurtz, 1960). The largest maize pollen grains are often located on the central spikes, and the smallest on the lateral spikes.

Pollen grains in general, range in size from 7 to 200 µm (Mildenhall et al. 2006). Maize pollen grains in particular, are relatively large compared to other grass pollen. They measure of about 70 to 125 µm in diameter (see table 3) and are among the largest particles that are commonly airborne (Raynor et al., 1972).

The weight of pollen grains among different plant species varies significantly from 13.4 ng per grain for oilseed rape (Fonseca, et al., 2003) to 250-882 ng per grain for maize (table 3).

Table 3 Summary of literature data on maize pollen size and weight

Size		Weight	
diameter, µm	**reference**	**ng**	**Reference**
70 - 100	Jones and Newell, 1948	250	Goss, 1968
94 - 103	Baltazar et al., 2005	210	EURL-GMFF: verification report for extraction of DNA from pollen in honey, 2012
76 – 105 81 – 100 80 – 103	Aylor , 2002	500	Porter, 1981
90 – 125	Eastham and Sweet , 2002	882 ± 2.2	Babendreier et al., 2004
70 - 90	Vaissiere and Vinson, 1994	700	Jarosz, 2003

At the time of harvest, fresh maize pollen has a water content of about 50% to 65% (Knowlton, 1921). Fonseca and Westgate (2005) reported similar data for pollen water content at around 57% during the initial hours of pollen emission. The authors also pointed out that corn pollen dries out rapidly in an atmosphere of relatively low humidity. The average moisture content of the maize pollen and its standard deviation are also determined by Vaissiere and Vinson (1991) as 45.7 ± 6.2%. Vinson (1927) reported 3.97% water in air-dried pollen. The specific gravity of fresh pollen can be less than one and varies considerably with the taxon and the environment (Brush and Brush, 1972). Pollen water content affects pollen mass, diameter and density. Marceau et al., 2012 determined that the maize pollen shape changes from spheroid to prismatic at a water content threshold of 25.6%. If water content decreases below 30% maize pollen loses its viability.

The effect of increased temperatures on the weight, size and atomic H/C ratio of pollen particles was examined by Ujile Y. et al. (2003) by heating living pollen grains of *Pinus thunbergii* to 290°C. At 136°C they measured a 22.8% loss in weight, about a 4% decrease in size and a decrease of about 5% in atomic ratio C/H. They did not detect changes in the C/N ratio, which shows that very minor compositional changes took place in the pollen grain at that temperature of heating (135°C) for water insoluble matter determination (Lord W.D. et al., 1988).

3.1.3. Qualitative information on harvested maize pollen

Pollen is the most important protein source for honeybees. Adequate pollen supply is essential to ensure the long-term survival of a colony and to maintain its productivity. Pollen provides honeybees with protein, minerals, lipids, and vitamins (Herbert and Shimanuki, 1978). Compositional variability in the quality of pollen and its nutritional value for honeybees, as well as the availability of pollen, depends on the floral origin and time of the year, correlated with the flowering periods of plants attractive to honeybees (Levin and Haydack, 1957; Standifer, 1967; Keller et al., 2005; Höcherl et al., 2012).

Maize pollen is usually only an extra food source for honeybees. When other valuable pollen sources are readily available honeybees do not show great interest in maize fields (Crane, et al. 1984 and Sabugosa-Madeira et al., 2007). However, maize tassels are often visited by honeybees for pollen collection (Maurizio and Louveaux, 1965), especially during the peak maize flowering time during early summer in France (Louveaux, 1958).

Pham-Delegue and Cluzeau (1999) placed beehives near sunflower field trials in Vendée, France to test the effects of pesticides on honeybee colonies. Samples from pollen traps showed that sunflower pollen was dominant during the flowering period of this crop, but maize pollen was also detected. In some samples maize pollen was even the dominant pollen species. This last observation was confirmed for France by Odoux et al. (2004).

In periods of poor flowering of melliferous plants, maize pollen could become a major source of pollen nutrition for honeybees (Höcherl et al., 2012), and pollen from maize plants is readily collected if other floral sources are limited (Wille and Wille, 1984; Krupke et al., 2012). Such observations were reported previously by Ibrahim (1976), Shawer (1987) and Atallah et al. (1989). During the spring time, when is scarce of pollen supply in the Assiut area of Egypt, Hussein (1982) also identified maize pollen as an important pollen source for honeybees after *Vicia faba*, *Trifolium alexandrinum* and *Brassica sinapis*. For the same conditions of short supply, but in Ghana, Amoako and Pickard (1999) reported that maize pollen becomes an important part of honeybees' diets.

Nowakowski and Morse (1982) conclude that maize pollen abundance is the main reason for honeybee visits, and thus constitutes its significant potential as a food source for honeybees. This was confirmed in Quebec in early August by Pion et al. (1983) and in Newark, Delaware from mid-July to mid-August by Mason and Tracewski (1982).

Keller et al. (2005) reviewed data for 40 years (1947-1987) on the percentage of pollen species collected from honeybees at one location in England, several in Scotland, three in Italy and seventeen in Switzerland. Maize was one of the six most frequently found pollen species, which on average made up more than 60% of the totally collected pollen. Even in earlier studies it is evident that agricultural crops (*Zea may*, *Trifolium repens, Trifolium pratense*, and *Brassica napus*) are important pollen sources for honeybees. Unfortunately, in most of the listed studies, information about the vegetation in the vicinity of the beehives is not reported. Nevertheless, a direct relationship between pollen availability and colony development can be expected, but honeybee colonies differ in their use of the available pollen at a given location (Moezel et al., 1987).

When beehives were located in areas with large maize fields with an experimental design in San Paulo, Brazil, honeybees fed almost exclusively on maize pollen (Malerbo-Souza, 2011).

3.1.4. Quantitative information on harvested maize pollen

Quantitative information for maize pollen collected by honeybees in the USA provided by Flottum et al. (1983) revealed that 25-55% (for the year 1980) and 30-40% (for 1981) of the total harvested pollen was maize pollen. Again for the USA, Erickson et al. (1997) reported that 2% to 18% of the total pollen collected by honeybees was maize pollen in 1982, and 4% to 25% for year 1983. The variability in maize pollen collection mainly reflects the differences in variety and climate conditions, resulting in differences in maize pollen abundance and attractiveness compared to pollen from other plant sources available at the same time. Krupke et al. (2012) also reported for USA, the state of Indiana that maize pollen comprised over 50% of the pollen collected by honeybees (by volume) in 10 out of 20 samples. The sampled beehives were located in completely intensified agricultural environments, with large fields of maize and soybeans, where other floral sources are significantly limited.

Pechhacker (2003) reported on the pollen intake of honeybees in Austria, showing that maize pollen presence made up to 50% of the total. Maize pollen was an important pollen source for honeybees. The intake of maize pollen varied considerably during the day between a minimum of 1.19% of the total pollen at late afternoon and a maximum early in the morning of 63.04%.

In 2007, Rosenkranz (2008) monitored the foraging of eight honeybee colonies placed up to 1 km from maize fields in Baden-Württemberg. In general, it was observed that the amount of maize pollen entering the beehive decreased with an increase in distance from the maize field, but GM maize pollen was still detectable at a distance of 1 km.

Hofmann et al. (2010) presented changes in the Bt-maize pollen content of the total harvested pollen by increasing the distances between beehive and maize fields from 100 m (during 2007) to 250 m in a northerly direction and 500 m in a westerly direction (during 2008). In 2007 for a distance of 100 m, the Bt-maize pollen content ranged from 3% to 49%. In 2008 at a distance of 250 m in a northerly direction and 500 m in a westerly direction the Bt-maize pollen content decreased to 1.9% of the total pollen.

In all studies pollen intake into the hive was estimated by using pollen traps that remove pollen grains from some of the returning foragers as they enter the hive. The percentage of retained pollen in a trap may be quite variable, but will always be considerably less than 100% (Waller, 1980). Extensive observations by Imdorf (1983) showed that the collection efficiency of traps on one colony can vary between 3% and 25%. Such discrepancies may result from small differences in the material of the nets used for the individual traps. Moreover, honeybee colonies may vary in the average size of the workers or may collect a different spectrum of pollen types. The species composition of the collected pollen appears to be of particular importance. Maize pollen grains are one of the largest pollen grains (section 3.1.2). Assuming that large pollen grains preferentially stripped off, the reported values likely overestimate the maize pollen share.

Therefore, accurate estimation of the actual quantity of pollen collected by a colony and its composition is virtually impossible using pollen traps. The situation is further complicated because colonies may change their behaviour in response to continuous pollen trapping, for example by increasing their foraging effort (Levin and Loper, 1984). It is also not clear to what extent honeybee colonies might be affected by extended use of pollen traps.

Most studies reviewed in this section are specifically designed to reveal the possible exposure of honeybees to pesticides and to assess the efficacy of different management procedures to reduce this exposure. Therefore, their relevance for determination of maize pollen presence in honey could be limited due to sampling strategy, location of examined beehives and sample quantity. Nevertheless, in the absence of studies specifically designed for the purpose of this document, these studies can at least provide an initial overview of the maize pollen percentage in the total of collected pollen per hive.

All the aforementioned data on maize pollen harvested by honeybees are summarized in table 4.

Table 4 Available quantitative information on maize pollen harvested by honeybees

Location and plant environment	Harvested maize pollen, (% of total pollen)	Reference
agricultural area, USA	25 - 55% (for 1980) 30 - 40% (for 1981)	Flottum et al., 1983
agricultural area, USA	2 - 18% (for 1982) 4 - 25% (for 1983)	Erickson et al., 1997
agricultural area, maize fields, state of Indiana, USA	> 50% (by volume, in 10 out of 20 samples, 10th and 12th May 2011)	Krupke et al., 2012
Austria	up to 50% differences during the day 1.19% (at late afternoon) - 63.04% (early in the morning)	Pechhacker, 2003
Maize fields, Baden-Württemberg, Germany	the amount of maize pollen entering the beehive decreased with an increase in distance from the maize field, but is still detectable at a distance of 1 km.	Rosenkranz, 2008
agricultural area, maize fields, Germany	3 - 49% (for 2007; Bt-maize pollen, 100 m distance from hive to the maize field) 1.9% (for 2008; Bt-maize pollen, 250 m distance in a northerly direction and 500 m in a westerly direction from hive to the maize field)	Hofmann et al., 2010

3.2. Pollen content in European produced honey and quality standards

3.2.1. Entry routes of pollen in honey

Pollen grains are usually present in floral nectar, which is considered as primary source of pollen intake in honey (Von der Ohe, 2011). When a honeybee lands on a flower in search of nectar, some of the flower's pollen is dislodged and falls into the nectar that is sucked up by the honeybee. At the same time, other pollen grains often attach to the hairs, legs, antenna and even the eyes of visiting honeybees. Collected nectar and honeydew are stored in the honey stomach. A large proportion of the pollen grains, contaminating nectar or honeydew are filtered out before the honeybee arrives at the hive and unloads the remaining contents of its honey stomach to other honeybees for use in the hive. The filtering process is particularly efficient in the case of large pollen grain size, as is the case with maize pollen (Bryant, 2001). In the hive the collected nectar and the rest of contaminating pollen will be regurgitated and deposited into open comb cells.

A secondary pollen entry in honey occurs when honeybees groom their body in an effort to remove entangled pollen on their hairs. During this process pollen can fall into open comb cells or into areas of the hive where other honeybees may transfer it into regions of the hive where unripe honey is still exposed in open comb cells. Some worker honeybees also collect pollen for the hive. The worker honeybees collect pollen with their front and middle legs and then deposit it in their "pollen basket" or orbicular (Snodgrass and Erickson, 1992). The pollen is stored inside the hive separately from the nectar cells (Almeida-Muradian et al., 2005). Nevertheless, during the process of depositing, some of the collected pollen can fall into the hive or into open honeycombs. Some of the stored pollen from previous year could remain in the hive to the next season and comprise an additional source for admixture, because worker honeybees occasionally might add pollen to the nectar they are transforming into honey by mistake. However, in general honeybees try to keep pollen from pollen loads separated in specific pollen combs for use later as a food source for brood rearing.

Additionally, airborne pollen, such as maize pollen, can be blown into a hive by wind although not in large amounts away from source fields.

During the uncapping of combs and honey extraction, pollen cells can be disturbed and a few pollen grains or parts of the stored pollen from the pollen cells may drop into honey. It is known as a third cause of pollen entry into honey (Von der Ohe, 2011). This incidence depends also on colony management. In Europe, usually honey supers are well separated from brood chambers and such pollen contamination of honey is extremely rare.

3.2.2. Quality standards for honey in respect of pollen content

The presence of pollen in the final honey marketed to consumers is also addressed by the quality standards required by European and international organisations. In Europe, honey quality criteria are specified in Directive 2001/110/EC and in the *Codex Alimentarius* standard (Codex Alimentarius Commission 2001).

The main goal of honey quality standards is to ensure that honey is authentic with respect to a number of requirements. Honey shall not contain any food ingredient other than honey itself nor shall any particular constituent be removed from it. Honey shall not be tainted by any objectionable matter. The authenticity of the botanical origin of honey is determined by sensory analysis, pollen analysis and several physicochemical methods while traditional melissopalynological methods are employed to test the geographical authenticity.

An important purity requirement for marketing honey in the EU is the limit of water-insoluble content. Water-insoluble matter in honey includes pollen, honeycomb debris, bee and dirt particles. Mandatory limits for it (stated by the *Codex Alimentarius* standard for honey – CODEX STAN 12-1981 and Council Directive 2001/110/EC) are fixed at no more than 0.1g per 100g, with the exception of "pressed honey" for which the limit is 0.5g per100g.

Pressed honey, harvested by pressing the combs, was a significant part of global honey production some time ago. However, nowadays almost all commercial honey is harvested by centrifugation. The threshold of 0.5% for water-insoluble content in pressed honey reflects the specificity of the utilized harvesting technique.

Standards specify that the water-insoluble content of honey shall be measured by the filtration of a honey solution in a glass crucible with a pore size of 15 to 40 µm. The maize pollen grains have an average diameter of 70 to 125 µm (table 3). Therefore any maize pollen grains present in honey will remain in the crucible and will be measured as part of its water-insoluble content, which should not exceed 0.1% of the total mass of honey, or for pressed honey - 0.5%.

The quality criteria in place, for organic honey are the same as for the conventionally produced one. The Commission Regulation (EC) No 889/2008 laying down detailed rules for the implementation of Council Regulation (EC) No 834/2007 on organic production refers only to conditions and control of organic honey production. It addresses specific requirements and housing conditions in beekeeping and does not specify additional quality criteria for organic honey.

For other bee products, quality standards are being researched and developed. For example, the currently ongoing FP7 project APIFRESH (with duration from 2010-07-01 to 2013-06-30) aims to develop European quality standards for other beehive products like bee pollen and royal jelly, including their safety and authenticity. Research and development activities include also analytical methods to determine the sensory properties, microbiological load and chemical composition of the specified products and methods of melissopalynology.

3.2.3. Pollen content in European produced honeys

A large amount of quantitative data on melissopalynological analysis of European uni- and poly-floral honeys is summarised in this section. These studies were performed mainly to check the botanical origin of honey and the quality for consumers.

Pollen grains are always found in natural honey processed using standardised methods. The pollen content of honey not only reflects regional agricultural practices and plant vegetation, but also the floral diversity and species composition of the plants foraged by honeybees, available in the vicinity of apiary (Louveaux et al., 1978).

In Europe more than one hundred botanical species can give unifloral honeys. Most of them have only a local prominence importance and are thus marketed on a limited scale, whereas others are part of the import-export market between different European countries (Persano Oddo et al., 2004).

In 1998 the International Honey Commission (IHC) created a working group with the aim of collecting representative analytical data for more than 30 physicochemical parameters related to the main European unifloral honeys. A total of 6719 honey samples produced in 21 countries of the European geographical area were examined (Persano Oddo and Piro, 2004) and in addition an extensive bibliographic review was performed (Piazza and Persano Oddo, 2004). The fifteen selected honey types of this working group are the most important in terms of abundance of production or commercial relevance in European countries. Table 5 summarizes and cross links data from experimental work and bibliographic searches for the total pollen grain content in these main European unifloral honeys.

Table 5 Total pollen content in main European unifloral honeys

Honey type	Pollen content						Water, (g ± SD[2])/100g (Persano Oddo et al., 2004)
	Persano Oddo et al., 2004			% of pollen in honey[3]	Piazza and Persano Oddo, 2004		
	No of data	Absolute number, (PG[1] ± SD[2])/g	Mean of specific in total pollen, %		No of references	Absolute number, PG[1]/g	
Brassica napus L. and “turnip rape”	52	7570 ± 3730	82.8	0.010± 0.005	1	>10000	17.0 ± 1.1
Calluna vulgaris (L.) Hull	14	5000 ± 4230	37.0	0.012 ± 0.010	1	10000 - 50000	18.5 ± 1.5
Castanea sativa Miller	257	28820 ± 18010	94.5	0.008 ± 0.005	1	>10000	17.5 ± 1.2
Citrus spp.	142	1050 ± 550	18.6	0.003 ± 0.002	3	<2000	16.6 ± 1.1
Eucalyptus spp.	110	26960 ± 13670	94.8	0.010 ± 0.005	2	>10000	16.0 ± 1.0
Helianthus annuus L.	92	1880 ± 1210	56.7	0.004 ± 0.003	2	<3000- <5000	17.8 ± 1.1
Lavandula spp.	84	820 ± 590[4]	8.2	-	1	<2000	16.7 ± 0.7
Rhododendron spp.	65	1260 ± 640	38.6	0.008 ± 0.004	1	<2000	16.6 ± 0.9
Robinia pseudacacia L.	226	920 ± 500	28.1	0.003 ± 0.001	1	<2000	17.1 ± 1.3
Rosmarinus officinalis L.	44	940 ± 390	28.7	0.004 ±0.002	1	<2000	16.4 ± 1.4
Taraxacum officinale Weber	44	3360 ± 1530	17.2	0.013 ± 0.006	1	<6000	16.2 ± 1.1
Thymus spp.	210	2590 ± 1790	36.0	0.008 ± 0,005	1	<2000	15.9 ± 0.9
Tilia spp.	37	1580 ± 960	22.9	0.006 ± 0.003	2	<2000; 1100±560	16.9 ± 1.2
Honeydew honey	78	15180 ± 11200[5]	1.5[6]	-	1	variable	16.1 ± 1.2
Honeydew honey from Metcalfa pruinosa (Say)	37	9030 ± 5370[4]	4.8[6]	-	1	variable	15.9 ± 0.7

[1] Pollen grains;

[2] Standard deviation;

[3] Weight fractions are calculated by equating the pollen grain shape to the sphere and assuming specific gravity of 1.0. The average diameters of pollen grains are retrieved from the Austrian melissopalynological website (www.paldat.org). The % of specific pollen is considered to account frequency of pollen grains distribution in honeys. For estimation of the rest of the pollen grain content the mean pollen grain with an average diameter of 41 µm is used (for details see p.30).

[4] Specific pollen;

[5] Total number of honeydew and pollen elements;

[6] HDE/PG, HDE-honeydew element

In general a nectar honey is considered as unifloral, when pollen of a given botanical origin is predominant and exceeds 45% (Crane, 1975 and Von der Ohe et al., 2004). If there is no predominant pollen the honey is classified as multi-floral.

However, the results of the pollen analysis cannot be always interpreted in this way. The relation between the percentage of certain pollen and the presence of the corresponding nectar is valid for normal pollen, but it has to be modified for under-represented and over-represented pollen. This is because, in the case of under-represented pollen, the quantity of nectar actually participating in honey formation is superior to what one would have expected from the pollen count, and in the case of over-represented pollen it is less[11].

The under- and over-represented honey varieties have a total pollen content which is, respectively, inferior and superior to those of normal honeys. Therefore EU produced poly-floral honeys with 6250 – 12190 (Ramos et all., 1999) and 2000 - 10000 (Van der Ham et al. 1999) pollen grain content in a gram of honey falls in the range defined by the most abundant EU unifloral honeys (820 – 28820) (table 5). On the basis of this distribution of the total numbers of plant (pollen and honeydew) elements in the currently produced and marketed honeys, Von der Ohe et al. (2004) proposed honey classification in five classes. The multi-floral honeys, honeydew honeys and mixtures of flower and honeydew honeys are categorized in the second class with 2100 to 10000 plant elements in one gram of honey. The first class includes unifloral honeys with under-represented pollen, containing less than 2000 plant elements per gram of honey and the third class covers unifloral honeys with over-represented pollen and honeydew honeys, with 10100 – 50000 plant elements per gram of honey. The forth and fifth classes include: unifloral honeys with strongly over-represented pollen and some pressed honeys; and almost only pressed honey respectively.

The empirical data for the total number of pollen grains in EU produced honey (table 5) can be converted into a weight fraction by equating their shape to a spherical one (with averaged diameter) and assuming a specific gravity of 1.0. In addition to this assumption, the common frequency of pollen grains distribution by size presented in honey should be considered. Dessein et al. (2005) reported that the majority of plant species have pollen grains in the range of 20 – 40 μm. Based on this finding and data on the abundance of botanical species exploited for honey production in Europe (Persano Oddo et al., 2004; Von der Ohe et al., 2004 and Laube et al., 2010) can be assumed the pollen grain distribution by size of : 80% (20 - 50 μm) + 15% (50 - 70 μm) + 5% (70 - 100 μm). As a result the average diameter of the mean pollen grain is estimated as approximately 41 μm, which is equal to the 36 ng in weight. In this case, based on the range of data given in table 5, the total pollen mass would be between 0.003 – 0.104 % of the total honey weight. Even if we assume that all pollen grains contribute to the water-insoluble matter of honey, the calculated total pollen mass in EU honeys would be well within the established legal threshold of water-insoluble matter in honey of less than 0.1%.

3.2.4. Quantitative information on the presence of maize pollen in honey

The studies reviewed in the following paragraphs provide information about the actual presence of maize pollen (be it conventional or GM) in honey, focusing on studies conducted or ongoing in the EU (a summary is presented in Table 6).

Germany

In Germany, the Federal Office of Consumer Protection and Food Safety has launched a large scale survey about the German honey situation. Three Bee Research Centres in Germany (in Celle, Berlin and Mainz) are engaged in a survey on the occurrence of pollen of all the crop species in honey which have been cultivated in Germany as GM crops in field trials. The survey started at the end of July 2012, and data are not available yet (at the time of completion of this report).

Some results of GM field trials from the German federal states Bavaria, Saxony and Baden-Württemberg are already available.

Herrmann (2008) reported data from Bavaria for 2004 and 2005. Beehives were placed in a maize field and in the surrounding area up to a distance of 700 m away. In 2004, maize pollen was detected in 31 out of 36 honey samples and Bt-maize pollen was detected in 11 samples. For 2005, because of unfavourable weather conditions for maize and other dandelions and flowering plants, maize pollen was only detected in 17 out of 36 honey samples. However, Bt-maize pollen was not detectable in honey samples in 2005. The author states that the presence of Bt-maize pollen was easily detectable in pollen samples, even at trace level. The amount of maize pollen tended to decrease as the distance to the nearest maize field increased. However, the variability of data was high.

Mildner et al. (2011) compared honey samples from beehives placed in a Bt-maize field with those placed at a distance of 3 km away in Saxony during 2008**.** The pollen content in honey was 0.01-0.04% of the total weight. Maize pollen represented 0.2% (3 km distance) and 3.0-5.0% (within

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[11] *Castanea* honey, for example, is strongly over-represented and it has to contain more than 90% pollen from the species before it can be considered unifloral. Other over-represented pollen could be *Eucalyptus* (> 83%), *Brassica napus* (> 60%) and *Phacelia* (> 60%) (Von der Ohe et al., 2004). In honey coming from species with under-represented pollen, uniflorality is guaranteed by a percentage inferior to the 45% necessary for normal honeys; *Lavandula* honey is considered unifloral if it contains 5-10% of the pollen of that species and the same applies to *Tilia* honey; for *Robinia, Rosemary and Citrus* honey 10-20% pollen from the species is necessary, etc. (Serra-Bonvehí, 1989; Martínez-Gomez et al., 1993; Serra-Bonvehí and Ventura-Coll, 1995; Persano Oddo, 1995; Thrasyvoulou and Manikis, 1995; Perez-Arquillue et al., 1995; Seijo et al., 1997)

maize field) of the total pollen. However, Bt-maize pollen quantification was difficult since the amount of maize pollen was only slightly above the detection limit.

Additional data on maize pollen presence in honey are presented by Hedtke and Etzold (1996) and Von der Ohe (2011) reviewing the maize pollen content of honey produced in Germany. Hedtke and Etzold (1996) analyzed 200 honey samples from Brandenburg. Maize pollen was only found in 5.5% of honey samples. In 2% of honey samples maize pollen was classified as an important single pollen, i.e. comprising 3-15% of total pollen and in 3.5% of honeys it was classified as rare (<3%). Von der Ohe (2011) presents data from 157 honey samples from different parts of Germany and found that 11% of the samples contained traces of up to 0.2% maize pollen in the total pollen fraction.

Spain

The Veterinary Faculty of the Complutense University of Madrid and Marchamalo Agricultural Center, Guadalajara conducted a systematic, four-year (2008 - 2011) study of 86 honeys from all over Spain (Miguel-Fraile et al., *in press*). For 2008, 20 honeys were analyzed, for 2009 - 25 honeys, and for 2010 and 2011 - 21 honeys for each year. According botanical origin and pollen content, the studied honeys were classified as heather, lavender, brown, eucalyptus, sunflower, honeydew, multi-floral and bush honey. Three of them are from the packaging line and the rest are collected directly from the producers. The melissopalynological analysis of these honeys showed that 22 of them contained maize pollen. The maximum maize pollen content was 3.96% of the total pollen present in the samples. The PCR analysis of all 86 honey samples for GM maize pollen presence was negative. The sensitivity of the assay used is 0.05%.

Czech Republic

In 2011, the State Veterinary Inspection of the Czech Republic collected 14 honey samples from different parts of the country. Eleven of the sampled beehives were located at a distance of 100 m to 5 km from Bt-maize MON810 fields. The others were more than 5 km away from GM maize fields. The Bt-maize MON810 fields measured 50 hectares or more.

The collected honey samples were analyzed for pollen content and presence of plant- and MON810-specific DNA sequences. According to their pollen profile the honey samples were classified as honey from flowers, mixed honey, forest honey or lime honey. In none of the honey samples were maize pollen grains identified. The end-point and real-time PCR tests did not detect GM maize pollen in any of the samples.

The findings of the Czech study suggest that maize pollen is not frequent in honey. The experts from the Bee Research Institute pointed out that maize crops flower at the time when honeybees bring the last nectar to the hives. Therefore, it is unlikely that honey from this period could contain pollen of Bt-maize MON810.

The Netherlands

In the Netherlands, Bees@Wur, Plant Research International carried out a large scale monitoring program among 172 Dutch beekeepers in 2008 (Kleinjans et al., 2012). The monitored beekeepers were evenly distributed throughout the country. The honey samples were taken from beehives in June.

The pollen analyses of about 200 honey samples showed pollen grains of at least 50 plant families, including both wind and insect pollinated species. Rosaceae pollen (including apple) and *Brassicaceae* pollen (including oilseed rape) were very abundant. *Chenopodiaceae* (including *Beta*), *Solanaceae* (e.g. potato) and *Poaceae* (grasses, including maize) were also often present in the honey samples.

Maize pollen was found in only two out of 200 samples and in very small quantities. The percentage of maize pollen in the two samples was 1.5% and 4.2% of the total amount of pollen.

Low presence of maize pollen in honey samples in the Netherlands could be due to the large diversity of flowering plant species during the honey harvest, resulting in the increased likelihood of selective collection of pollen by honeybees. Also, Dutch beekeepers rarely harvest their honey during maize flowering because during this period nectar flow is poor. Although maize pollen may often be collected by honeybees, the low level of honey production probably results in maize pollen rarely showing up in honey harvested by beekeepers.

Denmark

The Danish Beekeepers Association also launched a broad study on the pollen collected by honeybees. The preliminary results demonstrated that the maize pollen fraction is low, but the data are not yet ready for publication. The study contains detailed information on the position of the sampled beehives as well as the location of the maize fields.

Two preliminary studies about maize pollen presence in Danish honey were carried out some years ago. Kryger (in a personal communication with Preben Holm) reported for 2004 about 48 honey samples collected from all over Denmark. Only three of them contained maize pollen. The Danish Beekeepers Association undertook a similar analysis and found that 5% of 150 honey samples contained maize pollen. In both studies the amount of maize pollen was below one percent of the total pollen. Although experts agree that these studies may not be conclusive since sampling was not undertaken in a consistent way, they at least give preliminary information before the 2012 study is completed.

Other studies

Data for maize pollen content in honey are also available for: Poland (Wroblewska et al., 2006; Wroblewska & Warakomska, 2009; Stawiarz, 2009; Stawiarz & Wróblewska, 2010), Greece (Tsigouri et al., 2004), Croatia (Sabo et al., 2011), as well as for Turkey (Dogan, 2008) and Argentina (Valle et al., 2007).

The Polish data set represents 480 samples of honey, taken from Opatów and Sandomierz counties, Sandomierska upland, North-Eastern and Lubelszczyzna region of the country. Among the pollen of non-melliferous plants maize pollen, depending on the region, had an average frequency of <10% to 60% in multi-floral honeys, 10-25% in Brassicaceae honeys, <25% in *Salix* honeys, and <25% in *Trifolium* honeys.

Tsigouri et al. (2004) presented 329 honey samples from different botanical and geographical origins in Greece. Maize pollen was found in 8% of pine honeys, in 20% of cotton honeys and in 2% of thyme honeys. The maize pollen percentage of the total pollen count was 1% to 3% and classified as trace.

Sabo et al. (2011) reported maize pollen presence in 4 out of 8 honey samples from Varazdin county in Croatia. The maize pollen percentage of the total pollen in three samples was between 0.5-3% and in one sample 6%.

Dogan (2008) analyzed the pollen content of 39 *Trifolium* honeys from 18 city centres of different regions in Turkey. Maize pollen was detected only in 3 honey samples. Its percentage of the total pollen was 1-5% and classified as rare.

Valle et al. (2007) examined 127 honey samples from the South and South-West of Buenos Aires province in Argentina. Only in 3% of the honey samples was detected maize pollen with a content of less than 3% of total pollen and classified as minor pollen species.

It can be concluded from the reviewed studies that even though maize pollen could be a main feed source for honeybees, especially for beehives located in the vicinity of large maize fields, its presence in honey is rather rare and maize pollen is mainly classified as a minor pollen species. The low number of maize pollen grains in honey is anticipated because most of the maize pollen collected directly by foraging bees from maize plants is used as feed for other bees including young bees and for brood cells, rather than for storage as honey.

Table 6 Maize pollen presence in honey

Country of origin	Number of samples analysed	Number of samples with maize pollen	Maize pollen content		Reference
			% of maize pollen in total pollen	% of maize pollen in honey	
Germany	36	31 (86% of samples)	11 of them with detected Bt maize pollen presence (2004)		Herrmann, 2008
	36	17 (47% of samples)	Bt maize pollen not detected (2005)		
			0.2% (3 km distance) 3.0-5.0% (within maize field) (Bt maize pollen detection)	0.0000032% 0.00096% - 0.0012%	Mildner et al., 2011
	200	11 (5,5% of samples)	3 – 15% (for 2% of samples) <3% (for 3.5% of samples)	0.004%* - 0.046%* < 0.004%*	Hedtke and Etzold, 1996
	157	17 (11% of samples)	< 0.2%	< 0.0002%*	Von der Ohe, 2011
Spain	86 (2008 - 201 1)	22 (19% of samples)	with maximum of 3.96% (Bt maize pollen not detected)	with maximum of 0.006%* (Bt maize pollen not detected)	Miguel-Fraile et al., *in press*
Czech Republic	14	0	no Bt maize pollen detected		Report of State Veterinary Inspection of the Czech Republic
Netherlands	200	2 (1% of samples)	1.5%, 4.2%	0.0016%*, 0.0062%*	Kleinjans et al., 2012

Country of origin	Number of samples analysed	Number of samples with maize pollen	Maize pollen content		Reference
			% of maize pollen in total pollen	% of maize pollen in honey	
Denmark	48 150	3 (6% of samples) 8 (5% pf samples)	< 1% < 1%	< 0.001%* < 0.001%*	
Poland	480	< 10% to 60% (for multifloral honeys) 10%– 25% (for Brassicaceae honeys) < 25% (for *Salix* honeys) < 25% (for *Trifolium* honeys)			Wroblewska et al., 2006; Wroblewska and Warakomska, 2009; Stawiarz, 2009; Stawiarz and Wróblewska, 2010
Greece	329	8% (of pine honeys) 20% (of cotton honeys) 2% (of thyme honeys)	1% - 3%	0.001%* - 0.004%*	Tsigouri et al., 2004
Croatia	8	4	0.5% – 3% (for 3 of samples) 6% (for 1 sample)	0.0005%* - 0.004%* 0.011%*	Sabo et al.,2011
Turkey	39	3 (8% of samples)	1% – 5%	0.001%* - 0.009%*	Dogan, 2008
Argentina	127	4 (3% of samples)	3%	0.004%*	Valle et al.,2007

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[*] Weight fractions are quantified on base of the calculated maximum pollen content in EU produced honeys being 0.104% (section 3.2.3) and the percentage of maize pollen in total pollen provided by authors. Such an approach of calculation defines better the range of variation than particular values. In this case the aim was to estimate well the upper limit of the range rather than to underestimate it.

The reviewed studies do not specifically reflect the situation for commercially marketed honey. Most of them analyse honey samples taken directly from the beekeepers before being packaged for sale to consumers. Only a very limited amount of honey or none at all is sold directly to the consumer immediately after harvest from the hive. Traditionally, after harvesting, honey is stored as bulk quantities. During the storage period, a process of natural separation of different constituents of honey takes place. In this multicomponent fluid a thermodynamic process occurs, namely sedimentation by gravity of solid particles such as pollen, honeycomb debris, bee and filth particles. The upper and sediment layers, where the technological impurities of honey are concentrated, are commonly discarded during the packaging of small consumer containers. In the same step some pollen grains are also removed. Therefore the maize pollen content of 0.2% to 6% in total pollen of honeys produced in the EU, presented in table 6, is likely an overestimation for commercial honey ready to be marketed.

Even in the case of the most extreme proportion of 15% maize pollen found in total pollen (Hedtke and Etzold, 1996), the corresponding weight fraction quantified by using the maximum calculated pollen content in EU produced honeys being 0.104% (section 3.2.3) adjusted for the percentage of maize pollen content in total pollen provided by the authors, is 0.046%. Such an approach of calculation defines better the range of variation than particular values. In this case the aim was to estimate well the upper limit of the range rather than to underestimate it, because of that the value of 0.046% can be read as a maximum of the possible maize pollen content in honey.

4. Detection of GM pollen in honey

At present, real-time PCR methods, are the most suitable for detection and quantification of DNA extracted from the total pollen in honey.

Pollen DNA extraction from honey is the first step of the analysis. To this respect, Waiblinger et al., 2012, published a multi-laboratory validated method for total DNA extraction from honey.

In addition, the European Union Reference Laboratory for GM food and feed (EU-RL GMFF) reported an intra-laboratory assessed method (EU-RL GMFF: verification report, 2012) for the extraction and analysis of the pollen DNA present in honey, including the isolation and analysis of genomic pollen DNA using real-time PCR on commercial honey samples and honey samples spiked with various levels of GM MON810 pollen. The study verified that:

- The honey pollen extraction protocol developed at the EU-RL GMFF is suitable for reproducible extraction of PCR-grade DNA from genetically modified maize pollen present in honey samples;
- The lowest spiked amounts tested in this study were 0.05% (w/w) GM pollen in honey corresponding to 119 GM maize pollen grains (taking 0.21 µg as the average weight of a maize pollen grain). Such level was reproducibly detected using the EU-RL GMFF protocol and the EU-RL GMFF validated real-time PCR methods. The quantitative PCR method for detection of maize event MON 810 (ISO/FDIS 21570:2005) has a limit of detection of 5 genome copies or ≤ 0.1% (w/w) and a limit of quantification of 10 genome copies or 0.1 % of GM MON 810 (mass fraction) in conventional maize;
- In honey samples purchased from the retail market, the presence of maize, rapeseed and soy pollen could be demonstrated when applying the EU-RL GMFF protocol combined with EU-RL GMFF validated/verified real-time PCR methods;
- The protocol presented in this report is suitable for the quantification and detection of maize event MON810 pollen in honey at the level of 0.1% (w/w) of the total (GM and non-GM) Maize pollen in the honey.

These results demonstrate that it is currently possible to determine in honey the amount of GM pollen of a specific crop (e.g. maize GM pollen/total maize pollen) using validated PCR methods. However, at the current state of the art of the technology a practical and robust PCR protocol able to quantify GM pollen relative to total pollen in honey is unavailable. The problem lies in the fact that in all honeys, even if classified as unifloral, the pollen fraction consists of pollen from several species.

5. Best practices for coexistence of GM maize and honey production

The Technical Working Group (TWG) for Maize of the European Coexistence Bureau (ECoB) analysed in 2010 the best practices for coexistence between GM maize crop production with non-GM maize (Czarnak-Kłos M, Rodriguez-Cerezo E, 2010). Here the analysis is extended to the coexistence between GM maize crop production and honey production in the EU. The TWG assessed if any further coexistence measure to those currently recommended in the previous document was required to limit adventitious presence of GM maize pollen in honey avoiding economic loses for producers.

For this review, the TWG understands that honey with an adventitious presence of GM maize pollen above 0.9% (w/w) of total product needs to be labelled according to Regulation (EC) 1829/2003, with possible economic consequences for the producers.

However, the TWG also considered that some operators in the food chain may demand lower thresholds for honey. Therefore a threshold of 0.1%[12] was additionally taken into consideration.

For the analysis of the potential of GM maize pollen to end up in honey, the TWG reviewed data (chapter 3.2) and considered the following main findings:

- The presence of maize pollen grains in the floral nectar collected by worker honeybees is quite limited as maize is not a nectar producing plant. Maize pollen grains could end up in the nectar of other flowers only as contamination, driven by gravity, wind and honeybees.

- Maize has large and smooth pollen grains which are filtered out of the honey crop (not regurgitated) before the honeybee arrives at the hive and unloads the remaining contents of its honey crop for the use of other bees in the hive. The filtering process is particularly efficient in the case of large pollen grains, such as maize pollen.

- The flowering of maize, which cans last 2-4 weeks, also determines the limited maize pollen presence in honey.

- Even though maize pollen could become an important feed source for honeybees in experimental situations or when beehives are located in the vicinity of large maize fields, its final presence in honey is rather rare and therefore is usually classified as minor pollen.

- Even though scout honeybees can fly several kilometers searching for pollen and nectar, such flying distances are not a regular foraging behavior of a honeybee colony to cover its daily nutritional needs of pollen. This is particularly valid for maize, which provides only pollen and is not a nectar producing plant. A rough estimation based on current knowledge of the flying distances covered by honeybees for maize pollen foraging could be in the range of a few hundred metres up to about 1 km. Current knowledge does not allow to establish a statistical relationship between pollen content in honey and distance of beehives to maize crops.

- The presence of pollen in marketed honey is also regulated by European and international quality standards. The mandatory limit for water insoluble matter in honey is fixed to 0.1g / 100g with the exception of "pressed honey"[13] , for which the limit is 0.5g /100g. Since the water insoluble content includes the maize pollen fraction of honey (given the size of maize pollen grains) in addition to pollen of other plant species, debris and bee parts, the TWG concludes that the eventual presence of GM maize pollen in honey, if any, will be very low (below 0.1%), and obviously below mandatory labelling thresholds. The same is true for organically produced honey as the quality standards are the same. In addition, organic honey has an "obligation of procedure" requirement to locate beehives in areas where in a 3 km radius nectar and pollen sources consists essentially of organically produced crops.

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[12] This figure is typically considered as the limit of quantification for GM maize (ISO/FDIS 21570:2005). A precise figure for the case of GM maize pollen will depend on the validation of an extraction method for isolation and analysis of the pollen DNA present in honey developed at the EU-RL GMFF (EU-RL GMFF: verification report, 2012)

[13] Pressed honey, harvested by pressing the combs, was a significant part of world production of honey some time ago. However, nowadays almost all commercial honey is harvested by centrifugation. The threshold of 0.5% for water-insoluble content in pressed honey reflects the specificity of the harvesting technique utilized. Since no reliable data for production of pressed honey, coexistence analysis is focused on the most produced world widely extracted honey.

This conclusion is supported by the examination carried out by the TWG of the empirical evidence provided by numerous finished and ongoing studies investigating the presence of pollen in general or solely maize pollen (even specifically GM maize pollen) in samples of honey marketed in the EU, reviewed in section 3.2.3 and 3.2.4. Available data indicate that total pollen presence in honey ranges between 0.003 to 0.1 % in weight. Considering the share of maize pollen in total pollen in honey the extrapolated figures for maize pollen in honey would be an order of magnitude lower. In any case, it is important to stress that studies aiming at detecting this trace-levels of maize pollen are usually carried out with melissopalynological analysis and counting of pollen grains, and that a routine DNA analysis based on validated PCR protocol able to quantify total pollen in honey is unavailable. Once such a method could be found, the maize pollen fraction as well as the GM-pollen fraction of the total pollen could be established.

In conclusion, the TWG maize of the ECoB, based on the analysis of the evidence summarised in this document concludes that no changes in the Best practice document on maize coexistence of July 2010 (Czarnak-Kłos M, Rodriguez-Cerezo E, 2010) are necessary and that the current practices in honey production and marketing in Europe are sufficient to ensure that adventitious presence of GM maize pollen in honey is far below legal labelling thresholds and even below 0.1 %.

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Abstract

The Technical Working Group (TWG) for Maize of the European Coexistence Bureau (ECoB) analysed in 2010 the best practices for coexistence between GM maize crop production with non-GM maize . In this document the analysis is extended to the coexistence between GM maize crop production and honey production in the EU. The TWG assessed if any further coexistence measure to those currently recommended in the previous document was required to limit adventitious presence of GM maize pollen in honey avoiding economic loses for producers. The terms of reference for this review are presented in Section 1. An overview of the structure of the honey-producing sector in Europe is given in Section 2.

The EcoB TWG maize held two meetings in June and November 2012 and examined state-of-art-knowledge from scientific literature, study reports and empirical evidence provided by numerous finished and ongoing studies looking at the factors determining the presence of pollen in general or maize pollen (even specifically GM maize pollen) in samples of EU produced honey. In addition to biological factors (related to honeybee behaviour and maize pollen characteristics) the TWG also analysed existing mandatory quality standards that impact the eventual presence of pollen in commercial honey. The review of this information (coming from a total of 136 references) is presented in a structured manner in Section 3 of this document. Finally, the TWG reviewed the state of the art and possibilities for the detection and identification of traces of GM maize pollen in honey (Section 4).

The analysis of existing information indicates that total pollen presence in honey ranges between 0.003 to 0.1 % in weight. Considering the share of maize pollen in total pollen found in honey, the extrapolated figures for maize pollen in honey would be around an order of magnitude lower. Nevertheless, it is important to stress that studies aiming at the detection/identification of this trace-levels of maize pollen are usually carried out with morphological identification and counting of pollen grains, and that a routine DNA analysis based on validated PCR protocol able to quantify total pollen in honey is unavailable. Once such a method could be found, the maize pollen fraction as well as the GM-pollen fraction of the total pollen could be established. In conclusion, the TWG maize of the ECoB, based on the analysis of the evidence summarised in this document concludes that no changes in the Best practice document on maize coexistence of July 2010 are necessary to ensure that adventitious presence of GM maize pollen in honey is far below legal labelling thresholds and even below 0.1 %.

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