Coexistence between transgenic MON 810 maize and hives: pollen and flour flow by pollinator bees and honey labelling

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Abstract:

In Portugal, in 2012 and 2013, the cultivation of GM maize covered 9278 ha and 8202.2 ha, respectively, representing approximately 6-7% of the total maize sowing areas. The large-scale cultivation of GM maize is not uniformly distributed along the country. It has its highest expression in the Centre-South regions. Maize is a cross-pollinating species and, preferentially, its pollen is wind-dispersed although it may also be insect-dispersed.

In 2011, the European Court of Justice decided upon the need to demonstrate that pollen is a natural constituent of honey rather than an ingredient. Later, in 2013, the European Parliament defined pollen as a natural constituent of honey. This decision avoided strong financial implications concerning the need of honey labelling whenever Genetically Modified (GM) pollen makes up more than 0.9% of the species pollen fraction, according to the Regulation (EC) 1829/2003. Contrarily, the presence of flour, which is not a natural component of honey but may occur when industrial mills are in the neighborhood of the hives, might be treated according to the legal labelling framework for ingredients. Therefore, there is a need to distinguish between pollen and flour. Currently, the detection/quantification of GM components in honey is done by real-time PCR. However, until now no procedure can distinguish pollen from other kinds of GM material. Our main goal was, therefore, to develop a reliable and accurate method to allow distinguishing pollen from flour in honey. We investigated the ability of quantitative real-time PCR together with plasmid calibrants, triploid maize seed endosperms and haploid maize pollen, to develop a new approach to differentiate between adventitious presence of GM pollen and adventitious presence of GM flour.

Plasmid calibrant certified for a 1:1 copy number ratio (transgene copy number in relation to an endogenous gene copy number) allow for the distinction between triploid and haploid tissues.

A seven point dilution series from a 2x106 plasmid copies/µL solution was used to establish two calibration curves, being one for the transgene and other for a species-specific gene. DNA was extracted from honey, originally obtained in a Portuguese GM free zone (Região Autónoma da Madeira), spiked either with pollen, flour or embryos from a hemizygous population having the female progenitor as the transgene donor. PCR efficiencies were of 93 and 94% for both reactions. As proof-of-concept, samples of national and imported honey, commercialized either at local producers or in the

supermarkets, were analyzed and the quantification results compared with the spiked honey samples. With this information available, correct estimation of the relative transgene copy number allowed for the distinction between pollen and flour.

We have adapted real-time PCR to fit into the requirements of GMO labelling regulations. This approach has considerable potential to evaluate escapes of maize flour and for the establishment of recommendations for the milling companies in order to minimize effective entry of flour into the hives. The procedure has been in-house validated for maize.

Keywords: Honey, GMO, contaminant, labelling, real-time PCR, plasmid calibrant.

JEL codes: Q18

1. Introduction

In 2011, the European Court of Justice decided that beekeepers must prove that pollen is a natural constituent of honey and not an ingredient (Court of Justice of the European Union, 2011). Pollen, in general, is the male gametophytes of flowers and contains approximately 10% of nectar and commercial honey is composed of pellets of pollen moistened with bees' mouth secretions which are composed of different enzymes (Campos *et al*., 2008). The presence of pollen in honey seems not to be controversial. Several studies were conducted on comparison of honey composition on the basis of pollen content, botanical origin, quality, antibacterial activity among other characteristics (Mercan *et al.,* 2007; Hermosín *et al*., 2003). Pollen is expected to represent only a very small fraction of the honey matrix. Depending on the author, total pollen content in honey varies from 0.003% to 0.104% (Piazza and Oddo, 2004; Kleinjans *et al*., 2012; Davison and Kershen, 2014).

After the first cultivation of GM crops in Europe, one of the earliest concerns of the European Commission (EC) was to enable consumers to choose between GM and non-GM organic or conventional products. This led to the establishment of labelling policies and procedures for GM food and feed. Labelling is mandatory except when adventitious or technically unavoidable presence of a GMO in a given ingredient remains below 0.9% of that ingredient (European Commission, 2003).

The official requirement issued by the European Court of Justice rose from the need to substantiate the exemption from label of honey containing genetically modified (GM) pollen, either authorized or non-authorized for food purposes, what, if approved, would have cost hundreds of thousands of Euros to bee keepers and to the final consumers. Indeed, if pollen was considered as an ingredient, according to the Regulation 1829/2003, all GM pollen would need to be labelled if making up more than 0.9% of the total pollen fraction or of the pollen with the same botanical origin (European Commission, 2003). Considering pollen as a natural component of honey then the reference matrix for labelling purposes is the honey itself.

In November 2013, contrarily to the first decision of European Court, it was published the final verdict of the European Parliament that defined pollen as natural constituent of honey (European Recommendation (EC) 787/2004). Consequently and accordingly to the

rules regarding labelling of GM food, any GM pollen produced from plants containing authorized events, has not to be labelled even if it makes up more than 0.9% of the pollen fraction as this will never be above 0.104% of the honey fraction (Piazza and Oddo, 2004; Kleinjans *et al*., 2012; Davison and Kershen, 2014). However, if the genetically modified organism (GMO) is not authorized or is still undergoing an authorization process, it is not considered as approved for human consumption and therefore, the honey cannot be marked in Europe. Additionally, if the GM material present in honey is flour rather than pollen, then it has to be considered as an ingredient or contaminant. In this case the labelling regulation has to be applied (European Commission, 2003; European Parliament, 2013).

In Portugal, in 2012 and 2013, the cultivation of GM maize covered 9278 ha and 8202.2 ha, respectively, representing approximately 6-7% of the total maize sowing areas (Carvalho & Mourão, 2012). The large-scale cultivation of GM maize is not uniformly distributed along the country. It has its highest expression in the Centre-South regions. In fact, 2215.3 ha were localized in Lisbon and Tagus Valley region and 5041.5 ha in the Alentejo province (MAM, 2013).

The cultivation of GM maize in Portugal follows, in first instance, the European rules. Therefore, only the MON 810 maize event is authorized. MON 810 transgene codes for the CRY1Ab pro-toxin, a *Bacillus thuringiensis* derived protein that has insecticide properties against the European corn borer insects *Ostrinia nubilalis* (Hübner) and *Sesamia nonagrioides* (Lefebvre). Additionally, the Portuguese decree-law 160/2005, from 21st September, regulating genetically modified varieties, ensuring coexistence with conventional crops and with organic production, describes the mandatory rules of coexistence to be implemented by those who are interested in cultivating GM MON 810 maize. In detail, the coexistence law explains the technical standards to avoid cross-contamination of non-GM fields, to mitigate the selection of more resistant insects, to trace and label the production. In addition to the use of certified seeds, the most important measure is the distance of isolation. A GM maize field must be 200 m far from a conventional maize production field and 300 m from an organic production field (Quedas and de Andrade, 2013). These distances proved to be adequate for this purpose. However, no reference distance was stated in order preventing the presence of pollen in hives and/or in honey as the mechanisms of pollen dispersal may vary (Arrit *et al*., 2007). Maize is a cross-pollinating species and, preferentially, its pollen is wind-dispersed although it may also be insect-dispersed. Honeybees, *Apis mellifera* L., are the most important pollinators.

Several studies indicate that maize pollen may be present in honey, especially when the hives are localized in the neighbourhood of large fields and when maize blooms earlier or later than other plants with more attractive pollen (Wróblewska *et al*., 2010). Honeybees may forage up to 2 Km (Ramsay *et al*., 1999). A more recent study, suggests an isolation distance higher than 1.1 Km between GM and conventional oilseed rape fields (Chifflet *et al*., 2011).

This seems to be more complicated when the presence of flour in honey which may occur when industrial mills are in the neighbourhood of the hives. Flour is not a natural component of honey. Thus, it has to be considered as a contaminant or ingredient. During the last years it was considered that PCR was not a helpful technique to separate pollen from other kinds of contaminations. However, we consider that it was not optimised sufficiently.

Therefore, the aim of our study was to investigate the ability of quantitative real-time PCR using plasmids calibrants together with the triploid maize seed endosperm and the haploid pollen, to get good estimates for the copy number of a transgene in relation to the copy number of a species specific gene as a mean to differentiate between unavoidable presences of pollen from a flour contamination. A good estimation of the copy number of a transgene in relation to the copy number of a species specific gene would be valuable for considering potential escapes of flour and for the establishment of recommendations for the milling companies in order to minimize effective entry of flour into the hives.

2. Material and Methods

2.1. Honey sample preparation

GM pollen was collected from MON 810 grown under greenhouse confinement during the summer of 2012 in the High School of Agriculture in Santarém (ESAS). The pollen was stored dry in falcon tubes at room temperature in the GMO laboratory from the National Institute for Agricultural and Veterinarian Research (INIAV, I.P.). The pollen donor plant was verified by real-time PCR for the presence of the MON 810 transgene.

Commercial honey was obtained in two different regions from Portugal. The honey to prepare the honey-spiked samples was purchased in Madeira as it is a GM free zone. The other two samples were purchased either at local producers in Salva-Terra, a region having intensive MON 810 GM grain production, or in the supermarket being imported and composed by European and non-European honey.

2.2. Honey spiked samples

Honey samples were spiked with GM MON 810 maize pollen (haploid). Five assays were performed in order to evaluate DNA extraction yield and pollen recovery. GM pollen mass fractions in relation to honey mass are presented in Table 1. This is of most importance to estimate the most approximate amount of GM pollen in commercial samples of honey and to decide upon labelling. Theoretically, the pollen mass fraction in honey is never above 0.5% (Piazza and Oddo, 2004; Kleinjans *et al.*, 2012; Davison and Kershen, 2014). Therefore, 0.45% was the highest pollen mass fraction in the honey samples spiked with GM pollen. Besides, 0.005% was the lowest pollen mass fraction, which corresponds to twenty times less the percentage referred in the Commission Regulation (EU) 619/2011 for those GM events having a pending authorization procedure or an expired authorization and therefore requiring labelling (European Union, 2011).
Additionally, three more samples were spiked with ground embryos (heterozygous) and ground seeds either having a GM female progenitor or a GM male progenitor. These spiked samples were prepared to validate the method. Honey samples spiked with smashed embryos are expected to test equally to honey spiked with pollen from MON 810 heterozygous plants whereas honey samples spiked with flour are expected to show either 34-39% or 57-66% depending on the transgene donor parent (Holst-Jensen *et al.*, 2006).

2.3. Honey sample DNA extraction

DNA was extracted according to the validated CTAB-based method (van den Bulcke *et al.*, 2012) with three minor modifications: 1) each test portion of 12.5 g of warm honey was kept separate from the others until the resuspension in approximately 0.5 mL of sterile water. Only after this step, the four portions were reuniting in a single 2.0 mL reaction tubes containing 100 mg of glass beads (Ø = 500 µm); 2), 40 µL **RNase A** (10 mg/µL) were always added to 1 mL of CTAB buffer and followed by incubation for 30 min at 65 °C with soft shaking; 3) an additional washing step with 500 µL of chloroform followed by mixing of approximately 30 s and centrifugation for 10 min at 13 000 g was added.

2.4. Reference materials/calibrants

The European Reference Materials ERM®AD413 (plasmid DNA containing 1 copy of a fragment from the MON 810 transgene and 1 copy of a fragment from the High Mobility Gene - *hmg* - as the species-specific gene) and ERM®AD415 plasmid (plasmid DNA containing 1 copy of a fragment from the NK603 transgene and 1 copy of a fragment from the *hmg* as the species-specific gene) calibrants at the nominal concentration of $2x10^6$ copies/ µL were used to prepare standard calibration curves (Corbisier *et al*., 2010; Jeynov et al., 2011).

To establish calibration curves, nine points serial dilutions was done from the commercial solutions either from ERM®AD413 or from the ERM®AD415, ranging from $5x10^5$ copies/ µL to 5 copies/ µL. Tris/EDTA (TE) buffer was used as dilution buffer. The preparation was done as the protocol stated in the Certification Reports for the Certification of plasmid DNA containing either MON 810 or NK603 maize DNA fragments (Corbisier *et al*., 2010; Jeynov *et al*., 2011). The dilution series were always prepared fresh. The same dilution series was used to prepare the two calibration curves, one for the transgene and one for the taxon-specific gene each having 5 points:

1) Endogenous gene for maize (*hmg*) quantification: $1x10^5$, $2x10^4$, $2x10^3$, $1x10^3$, $2x10^2$
2) Transgene (MON 810 or NK603) quantification: $1x10^4$, $2x10^3$, $2x10^2$, 2x10, 5

ERM®BF413f maize GMO standard for 5% MON 810, certified for its MON 810 mass fraction, was used as quality control for the evaluation of the PCR efficiency through the slope of a calibration curve established by means of a five series dilution. It was also used as positive control.

2.5. Quantitative real-time PCR

Real-time PCR screening methods and event-specific detection and quantification methods can be used on the extracted DNA from honey providing that enough DNA of PCR-grade is available. At first, the amplification of approximately 720 bp of the mitochondrial cytochrome oxidase I gene (Folmer *et al.,* 1994) is used as an internal control for the amplifiable ability of the extracted DNA. The method was applied without modifications.

Real-time PCR methods used for the detection and quantification of transgenes, events MON 810 and NK603, were obtained from the European Union Reference Laboratory for GM Food and Feed (EURL- GMFF), Joint Research Centre, Ispra, Italy (Joint

Research Centre, 2004 and 2005) with one significant modification: a plasmid calibrant certified for the copy number ratio was used rather than a reference material (CRM) certified for its GM mass fraction.

The real-time PCR quantification methods, based on TaqMan chemistry, were applied without modifications.

Each point of the calibration curve as well as each DNA extract were measured in triplicate in each the PCR reaction.

3. Results and Discussion

3.1. Real-time PCR quantification method

The use of the recent GMO Plasmid Calibrant Certified Reference Materials in accordance to the correct implementation of the European Recommendation (EC) 787/2004 together with the event-specific MON 810 and NK603 detection methods (Joint Research Center, 2004 and 2005) allowed for the relative quantification of a specific transgene copy number in relation to a maize specific gene originated either from pollen or from flour in honey matrix. The quantification is based on two different measurements calibrated by the same plasmid which was certified to contain one copy of both the GM and the taxon-specific target sequence (*hmg* for the maize case). As the *hmg* is a maize single copy gene, its copy number directly reflects the number of total maize haploid genomes. Similarly, as MON 810 and NK603 are single integration-site transgenes the relative GM DNA copy number reflects the number of GM haploid genomes.

PCR reactions showed high efficiencies (93-94%) and acceptable R2 coefficients (0.999-1.000) in accordance to the Minimum Performance Requirements for the Analytical Methods of GMO Testing (ENGL, 2009). The Limit of Detection (LOD) was calculated as [3.3 x titer of the calibration curve point x Standard Deviation of the triplicates of the calibration curve point]. On average 25 transgene copies was the obtained practical LOD.

3.2. Pollen extraction yield

As a reference value, 0.50 µg was considered the average mass of a maize pollen grain (van den Bulcke *et al*., 2012; Babendreier *et al*., 2004). Based on this assumption, the number of pollen grains was calculated for each pollen fraction used in the spiked samples

(Table 2) and measured by real-time PCR associated to plasmids calibrants. Slopes of real-time PCR calibration curves prepared for the detection of the transgene MON 810 and for the detection of the maize endogenous gene, *hmg*, by using the plasmid calibrant ERM®AD413 were very similar, 94.24% and 93.73% respectively, and within the range of the validated values (Figure 1) (Corbisier *et al*., 2010). The highest recovery was obtained for the 0.04% [m (pollen)/m (honey)] sample followed by the less "contaminated" samples. This result is directly linked to the DNA extraction procedure, as the recommended honey sample intake is 50 g divided in four subsamples of 12.5 g. This means that for the 50 g honey-spiked samples, although having the highest pollen concentration, DNA had to be extracted from 4 sub-samples increasing the losses and the errors associated.

3.3. Pollen and flour quantification

Maize seeds comprise a large triploid endosperm where two haploid genomes are mother-inherited and one is father-inherited. Therefore, when the female progenitor is the transgene donor, for the same seed mass, it is expected to detect more transgenes than when the transgene donor is the male progenitor. According with Holst-Jensen and co-authors (2006) the transgene relative quantification values for GM seeds having the female progenitor as a transgene donor are expected to be between 57% and 66% whereas for seeds having the male progenitor as the transgene donor would range from 34% to 39% (Holst-Jensen *et al*., 2006).

In order to differentiate pollen from flour, we made use of the different ploidies intrinsic to different maize plant tissues: embryos – diploid; pollen- haploid; seed flour- mixture of haploid, diploid and triploid. Embryos excised from hemizygous seeds have only one transgene copy in each diploid cell, therefore, 50% is the expected value for a GM relative quantification in relation to an endogenous species-specific gene. Additionally, a hemizygous plant produces 50% GM and 50% non-GM pollen. Theoretically, it is expected a GM quantification result similar to that of embryos. Finally, GM quantification results obtained with ground seeds would follow the values reported by Holst-Jensen and his co-authors (Holst-Jensen *et al*., 2006). Therefore, GM-free honey spiked with material having different ploidies is expected to exhibit different and tissue-specific transgene content.

Results of samples spiked with ground embryos, pollen and ground seeds (Table 3) illustrate the above mentioned situations and, therefore, demonstrate that our novel real-time PCR associated with plasmid calibrants approach is applicable to plant species having seeds with endosperms either with different ploidy (e.g. maize) or with a different genetic composition (e.g. sugar beet) from the pollen.

3.4. Analysis of commercial honey samples

As a proof-of-concept, we have applied our novel approach to samples of commercial honey mimicking the routine analysis for the official food controls. Two honey samples were analyzed. One was obtained at a local producer in Salva-Terra, an area of intensive MON 810 GM cultivation in Portugal, and the other was imported and purchased in a local supermarket. The latter was composed of European and non-European honey. In a previous quantification analysis using a certified reference material for the mass fraction (ERM®BF413f), both samples tested positive but it was not possible to identify the source of GM material.

Using our novel approach, the honey produced in Salva-Terra quantified 52.63% (copy number ratio) which corresponds to the presence of pollen (Table 4) and therefore, it falls out of the scope of the Regulation 1829/2003/EC (European Commission, 2003). The imported honey, purchased at a local supermarket, may have a flour contamination, as the quantification value is close to the value for flour originated from seeds generated from a GM male progenitor. However, it is still lower than the values expected for all situations but approximately half of the value expected for pollen. This sample tested also positive for oilseed rape. Therefore, it is of high probability that pollen from other GM maize event could be present. This would increase to the content of the hmg gene and, as a consequence, decrease the copy number ratio of the MON 810 maize. This sample was additionally tested for other GM maize being positive for NK603 event, frequently stacked with MON 810. In this situation no labeling is required as both events are approved for food uses.

4. Conclusion

The triploid maize seed endosperm, main constituent of flour, can be distinguished from diploid heterozygous embryos and haploid pollen grains by measuring the copy number of transgenes in relation to the copy number of a species-specific endogenous gene. The

simultaneous use of real-time PCR, plasmid calibrants and different ploidies has potential to differentiate pollen grains from flour in maize.

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Tables and figures

Table 1. Preparation of spiked honey samples; GM pollen mass fraction in relation to honey mass.

Spiked samples	GM pollen (mg)	Pollen GM/non-GM (%)	Honey (g)	GM pollen/honey (m/m)
1	225	50	50	0.45
2	50	50	50	0.1
3	5	50	12.5	0.04
4	0,75	50	10	0.01
5	0,5	50	10	0.005

Table 2. GM pollen grains expected and estimated through real-time PCR (by using 5 µL of DNA extract) and their correspondent recovery (%).

GM pollen (expected no. grains/real-time PCR reaction)	GM pollen (estimated number of grains	Recovery (%)
5625	1578	28.1
1250	622.8	49.8
550	525.7	95.6
75	50	67.2
45	40	89

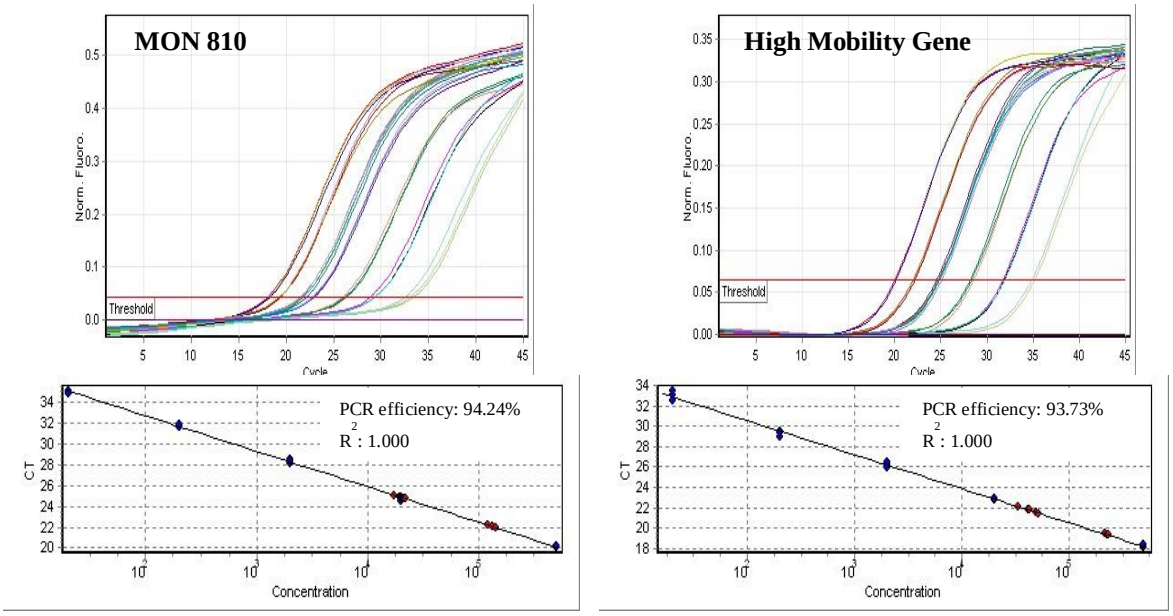

Figure 1. Real-time PCR reactions slopes, efficiency and linearity obtained by using plasmid calibrants.

Table 3. GM quantification results obtained in honey samples spiked with material having a different ploidies.

Matrix	GM % (copy number ratio) (Average ± Standard deviation)
Embryos (hemizygous)	53.73 ± 0.09
F1 seed (transgene donor: parent)	59.07 ± 0.02
F1 seed (transgene donor: parent)	38.83 ± 0.04
Pollen (from a hemizygous plant)	52.82 ± 4.25

Table 4. Quantification results on the commercial honey samples analyzed for the MON 810 event.

Matrix	GM % (copy number ratio)	Conclusion
Salva-Terra honey	52.63	GM pollen
Flowers honey	24.02	unknown

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