Quantitative Real-time PCR differentiation between Genetically Modified pollen and Genetically Modified flour in honey

Eugénia de Andrade[1*], Maria Lopes[2], Maria Clara Fernandes[1], Fátima Quedas[2], Isabel Rodrigues[1], Amélia Maria Lopes[1]

[1] UEISAFSV, Instituto Nacional de Investigação Agrária e Veterinária, I.P., Quinta do Marquês, 2780-159 Oeiras
[2]Instituto Politécnico de Santarém – Escola Superior Agrária de Santarém, Quinta do Galinheiro – S. Pedro, 2001-904 Santarém
***e-mail:** eugenia.andrade@iniav.pt

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 neighbourhood of the hives, has to be treated according to the legal labelling framework for ingredients. Therefore, there is an urgent need to distinguish between pollen and flour. During the last years no helpful technique was found to separate pollen from other kinds of contaminations.

In this work, we investigated the ability of quantitative real-time PCR, associated with plasmid calibrants and Taqman chemistry, to differentiate between an unavoidable presence of GM pollen from a GM flour contamination. A seven point dilution series from a $2x10^6$ plasmid copies/µL solution was used to establish two calibration curves, being one for the transgene and other for a species-specific gene. A good estimate for the relative transgene copy number was obtained for DNA extracted from honey from Madeira Island, a Portuguese GM free zone, spiked either with pollen, flour or embryos from a hemizygous segregating F1 population which had the female progenitor as the transgene donor. Spiked honey was used to minimize the matrix effect. PCR efficiencies were of 94%-99% for both reactions. This approach has considering potential to distinguish pollen from flour and, therefore, to help the national services for the control of labelling and to establish recommendations for the milling companies to minimize the entry of flour into the hives.

Introduction

Trace amounts of pollen are always present in honey. Some studies refer that they can range from 43 µg to 670 µg pollen/g honey (Kleinjans *et al*., 2012). The definition of pollen, as a fraction of honey, was under discussion for about three years. In 2014, pollen was considered as a natural constituent of honey rather than an ingredient. Consequently, according to the Regulation 1829/2003/EC:

1) honey having pollen from GM plants with authorized event(s), even if making up more than 0.9% of the species pollen fraction don't need labeling as pollen fraction is now related to the total honey mass and it comprises between 0.005 and 0.05% of honey (Davison and Kershen, 2014);
2) honey having pollen or flour from non-authorized GM plants or from GM plants undergoing an authorization process cannot be marked in Europe;
3) honey having flour from GM plants with authorized events need to be labeled if making up more than 0.9% of the flour fraction.

In Portugal, the coexistence between GM and non-GM maize production is ruled by the PT Decree-Law 165/2005, September the 21st. Although with proved success, this regulation does not mention reference distances to prevent either GM maize pollen or GM maize flour in hives/honey. Consequently, in order to fulfill with the labeling rules, there is a need to differentiate between an unavoidable presence of pollen from a flour contamination.

Thus, we investigated 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 distinguish between pollen and flour from maize.

Material and Methods

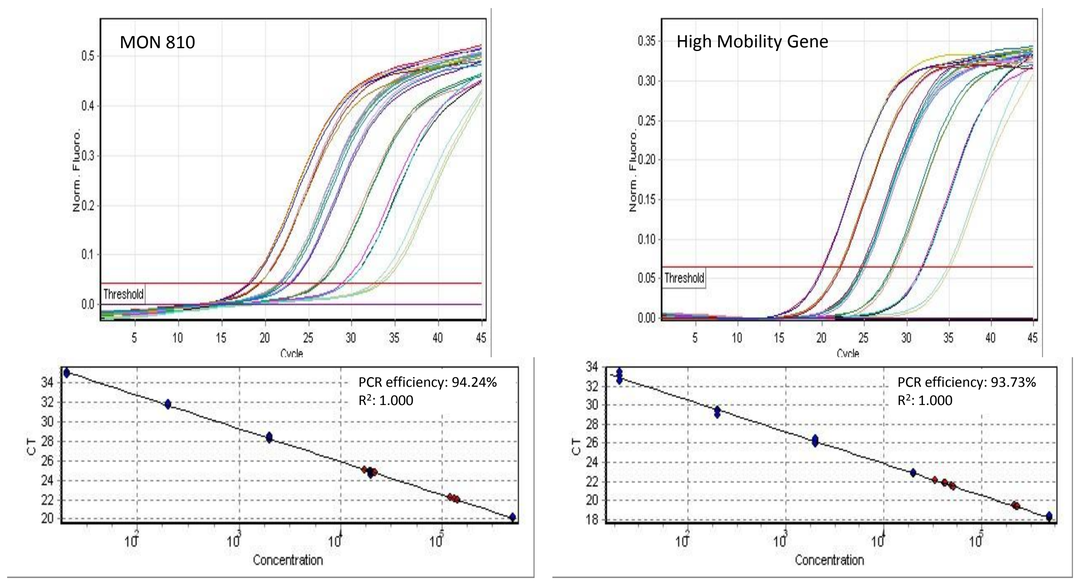
Pollen sample	MON 810	
Reference Materials	ERM®BF413f	
	ERM®AD413 plasmid calibrant ($2x10^6$ copies/µL) contains 1 MON 810 5' plant-P35S junction: 1 maize endogenous High Mobility Group gene	
Honey Samples	SV (Salva-Terra, Pt); Cor (Coruche, Pt);	
	MA (Madeira, Pt free zone) spiked with MON 810 pollen (P:H)	(225 mg:50g), (50 mg: 50 g), (3.75 mg:12.5 g), (0,75 mg:10 g), (0.45 mg:10 g))
	FL (Flores, Imported); LE (Leziria, Imported)	
DNA extraction method from honey	Report EUR 25524 EN - **DNA Extraction from honey and pollen – CTAB** (Van den Bulcke et al., 2012)	
Primers and probes	Reference method CRL-VL-25/04VR	
Calibration curves	To prepared the calibration curve, a serial dilution was done in 9 tubes ranging from 5×10^5 cp/µL to 5 cp/µL. TE buffer was used as dilution buffer. The preparation was done as described in the EUR Certification Report: 1) endogenous genes quantification: 10^5, $2x10^4$, $2x10^3$, 10^3, $2x10^2$ 2) transgene quantification: 10^4, $2x10^3$, $2x10^2$, 20, 5	

Results

Pollen recovery

GM pollen (expected no. grains/reaction)	GM pollen (measure nº of grains)	Recovery (%)
5625	1578	28.1
1250	622.8	49.8
550	525.68	95.6
75	50	67.2
45	40	89.0

PCR efficiency and linearity



Pollen *vs.* flour

Matrix	GM % (copy number ratio)
Embryos (hemyzygous)	53.73 ± 0.09
F1 seed (transgene donor: ♀ parent)	59.07*[1)] ± 0.02
F1 seed (transgene donor: ♂ parent)	38.83*[2)] ± 0.04
Pollen (from a hemyzygous plant)	52.82 ± 4.25

* According with Holst-Jensen *et al* (2006) the expected values are : 1) 57-66%; 2) 34-39%

Results on real honey samples

Matrix	GM % (copy number ratio)	Conclusion
SV honey	52.63	GM pollen
FL honey	24.02	GM flour

Conclusions

The triploid maize seed endosperm (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 Taqman chemistry has potential to differentiate pollen grains from flour in maize.

Literature

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