BOOK OF ABSTRACTS

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PP 6: Development of a new approach based on NADH dehydrogenase subunit 1(ND1) marker and high resolution melting (HRM) analysis towards the authentication of the geographical origin of honey

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Abstract: *In the present study, an approach for verifying the geographical origin of honey based on its entomological origin is proposed. The method was developed based on the polymerase chain reaction (PCR) amplification of a new markercontaining different single nucleotide polymorphisms characteristic of honeybees of different mitochondrial (mtDNA) lineages, therefore generating different fluorescent curves on HRM analysis. The method was successfully applied to honeys from Portugal and Italy, expected to be from lineages A and C, respectively, demonstrating their origin compliance.*

Keywords: honey authenticity, *Apis mellifera* subspecies, high resolution melting (HRM)

Introduction: Honey is a highly appreciated food worldwide being reported as one of the 10 food products in the European Union (EU) most prone of being adulterated [1]. Until now, honey authenticity was mainly focused on the issues of sugars addition and botanical origin. However, recently, an increased attention has been paid to the entomological origin of honey since it can also be related to geographical origin. In fact, different EU honeys labeled with protected designation of origin (PDO) must be produced with autochthonous *Apis mellifera* subspecies [2]. In particular, several PDO honeys from Portugal should be produced using only *A. m. iberiensis* (lineage A) while in the Italian region of Bologna rearing colonies other than *A. m. ligustica* (lineage C) is forbidden by law.

Experimental methods: The new set of primers targeting the ND1 marker were used to amplify different samples of honeybees belonging to *mt*DNA lineages A, M and C, and optimize the PCR conditions using the fluorescent dye EvaGreen. Assays were performed using 1 µL of sample (0.1 ng and 5 ng DNA for honeybees and honey samples, respectively), 1 × SsoFast™ EvaGreen® Supermix and 300 nM of each primer, and the conditions: 95 ºC for 2 min, 50 cycles at 95 ºC for 20 s, 52 ºC for 25 s and 60 ºC for 25 s, with collection of fluorescence signal at the end of each cycle. HRM curves were obtained from 58 ºC to 95 ºC with increments of 0.2 ºC every 5 s, with acquisition of fluorescence signal at the end of each melting temperature. Honey samples (25 g) were submitted to an optimized pre-cleaning step and DNA was extracted using the Nucleo Spin Plant II kit; extracts purity and concentration were determined by spectrophotometry. Samples of honey from Bologna (Italy) and Portugal were used to verify the proposed method.

Results and discussions: The optimized conditions of real-time PCR allowed establishing an absolute limit of detection (LOD) of 0.1 pg of honeybee DNA, a reaction efficiency of 103.4% and a R^2 of 0.997 (Fig. 1A and 1B). The developed HRM analysis allowed the successful differentiation of honeybees from lineages A, M and C in three different clusters with high percentage of confidence (>99%).When applied to honey samples analysis, the honeys from Portugal were

clustered with *A. m. iberiensis* lineage A while those from Italy were clustered with *A. m. ligustica* (lineage C), which is in good agreement with their geographical origin (Fig 1C).

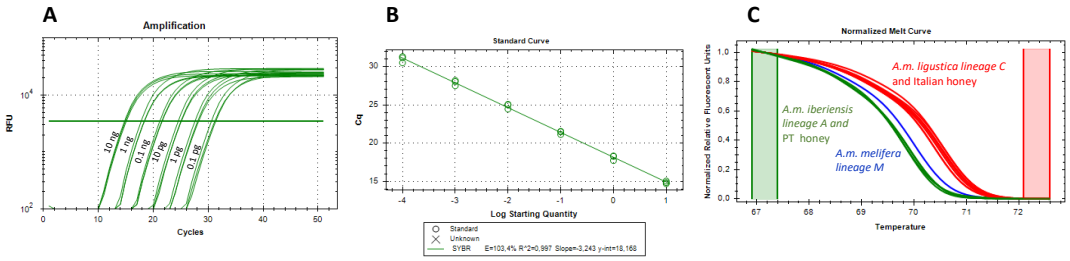

Fig. 1 Amplification (A) and calibration (B) curves of the optimized real-time PCR assay with EvaGreen® dye, targeting the ND1 marker using 10-fold serially diluted honeybee DNA (10 ng to 0.2 pg). Normalized melting curves (C) obtained by HRM analysis of voucher honeybees (lineages A, M and C) and honey samples from Portugal (PT) and Italy (IT).

Conclusions: A new approach based on PCR amplification with the fluorescent dye EvaGreen followed by HRM analysis is proposed to verify the labeling compliance of PDO honeys that should be produced with specific autochthonous honeybees subspecies.

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