



Analytical Methods

Automated DNA extraction from pollen in honey

Patrick Guertler^{a,*}, Adelina Eicheldinger^a, Paul Muschler^b, Ottmar Goerlich^a, Ulrich Busch^a^a Bavarian Health and Food Safety Authority, Molecular Biology, Veterinärstr. 2, 85764 Oberschleißheim, Germany^b Promega GmbH, High-Tech-Park, Schildkrötstraße 15, 68199 Mannheim, Germany

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ABSTRACT

In recent years, honey has become subject of DNA analysis due to potential risks evoked by microorganisms, allergens or genetically modified organisms. However, so far, only a few DNA extraction procedures are available, mostly time-consuming and laborious. Therefore, we developed an automated DNA extraction method from pollen in honey based on a CTAB buffer-based DNA extraction using the Maxwell 16 instrument and the Maxwell 16 FFS Nucleic Acid Extraction System, Custom-Kit. We altered several components and extraction parameters and compared the optimised method with a manual CTAB buffer-based DNA isolation method. The automated DNA extraction was faster and resulted in higher DNA yield and sufficient DNA purity. Real-time PCR results obtained after automated DNA extraction are comparable to results after manual DNA extraction. No PCR inhibition was observed. The applicability of this method was further successfully confirmed by analysis of different routine honey samples.

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1. Introduction

Bees process nectar with enzymes before converting it to honey by reducing the water content. Honey is used for human consumption, has medical benefits and is used for cosmetic purposes. However, honey is also a potential risk for human health due to environmental contaminations like pesticides, antibiotics, microbes or heavy metals (Corsini, Sokooti, Galli, Moretto, & Colosio, 2013; Martel et al., 2007; Nevas et al., 2002; Ortelli, Edder, & Corvi, 2004). Ingestion of pollen, as a characteristic component of honey, may also lead to allergic reactions (Helbling, Peter, Berchtold, Bogdanov, & Müller, 1992).

Therefore, honey is subject to morphological, chemical and molecular biological investigations. Analysis of floral origin by microscopic analysis of pollen (Louveau, Maurizio, & Vorwohl, 1978; Senyuva et al., 2009) is still state of the art. Laube et al. developed a DNA-based method for characterisation of the geographical origin of honey (Laube et al., 2012) and DNA-based methods may also be used to detect allergens and microbes in honey (Olivieri, Marota, Rollo, & Luciani, 2012).

Pollen from genetically engineered (GE) plants may also be present in honey. Until 2011, this had no effect on marketing issues or labelling of honey and honey products. However in September

2011, the Court of Justice of the EU declared pollen to be an ingredient and not a characteristic component of honey as before. As a consequence, honey that contains pollen of a GE plant, which is not authorised as food ingredient, must be removed from the EU market. In case of an authorised GE plant, the honey must be labelled above a GE content of 0.9% (Regulation (EC) No 1829/2003 on genetically modified food and feed, 2003a; Regulation (EC) No 1830/2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending 50 Directive 2001/18/EC, 2003b). Currently, surveillance of honey and honey products for GE contamination is performed by DNA analysis.

DNA analysis requires an efficient DNA extraction method in order to detect minute amounts of DNA, e.g. DNA from allergens or GE plants. Waiblinger et al. (2005) developed a manual CTAB buffer-based DNA extraction procedure, which was further optimised in 2012 (Waiblinger et al., 2012). However, this method is very laborious, contains the use of chloroform and requires a subsequent purification step by using a commercial kit. In order to resolve these negative aspects of this manual DNA extraction procedure, we modified a previously developed automated DNA extraction method (Guertler et al., 2013) to cope with honey as a very complex matrix. An extraction method for DNA from honey must feature a rapid and easy workflow and should result in DNA of high yield and quality. It is also essential to remove inhibitors in order to accomplish a reliable and reproducible PCR analysis.

* Corresponding author. Tel.: +49 9131 6808 5827.

E-mail addresses: patrick.guertler@lgl.bayern.de (P. Guertler), paul.muschler@promega.com (P. Muschler).

2. Materials and methods

2.1. Sample material

For assay validation, eight different honey samples (one pine honey, two wild honey, five polyflora honey) were pooled in order to gain enough sample material for DNA extraction and method validation. Honey samples were provided by the Bavarian Health and Food Safety Authority (Oberschleißheim, Germany). Further, twelve routine honey samples were analysed in order to check the applicability of the automated DNA extraction in the official honey surveillance.

Prior DNA extraction, honey was pre-warmed to 60 °C to permit easier handling.

2.2. Automated DNA extraction

Initially, a previously developed automated DNA extraction procedure was used for DNA extraction from honey (Guertler et al., 2013). Unfortunately, with honey as feedstock this procedure did not result in sufficient high quality DNA (data not shown). The DNA extract contained too many inhibitors, which led to the need of further optimisation of this method in order to isolate high quality DNA from honey.

We used the Maxwell® 16 FFS Nucleic Acid Extraction System, Custom-Kit (Promega GmbH, Mannheim, Germany) and altered the initial sample amount, the volume of CTAB extraction buffer, the volume of lysis and binding buffer. We also added different kinds and volumes of enzymes and other additives. This optimisation process led to an automated DNA extraction protocol that was validated using a pooled honey sample. The pooled honey sample was extracted 12 times and analysed by real-time PCR, subsequently. The results were compared with results from a manual CTAB buffer-based DNA extraction. In addition, the reproducibility was determined by extracting the pooled honey sample on three consecutive days.

2.3. Manual CTAB buffer-based DNA extraction

A CTAB buffer-based DNA extraction protocol, based on Waiblinger et al. (Waiblinger et al., 2012), was compared with the automated DNA extraction. For each honey sample ($N = 12$), four 50 ml Falcon tubes were filled with 12.5 g honey and 45 ml H₂O subsequently. Tubes were shaken until the honey was dissolved, followed by a centrifugation step for 15 min at $4.000 \times g$. The supernatant was discarded and the pellet was resuspended in 5 ml H₂O. Subsequently, all four tubes for each sample were pooled and filled up with H₂O to 30 ml total volume. After centrifugation for 10 min at $4.000 \times g$, the clear supernatant was discarded and the pellet was resuspended in 500 µl H₂O. This solution was transferred to a 2 ml tube prefilled with zirconium beads (Retsch, Haan, Germany) and incubated over night at 4 °C. On the next day, homogenisation was performed in a swing mill (Retsch, MM 301, Haan, Germany) with a frequency of 20/sec. for one minute. Subsequently, 1,000 µl CTAB extraction buffer and 18 µl RNase A (100 mg ml⁻¹) were added, followed by an incubation step at 65 °C for 30 min while shaking. After incubation, 30 µl Proteinase K (20 mg ml⁻¹) were added and samples were incubated at 65 °C for 60 min while shaking. Samples were centrifuged for 5 min at $10.000 \times g$. One thousand µl of the clear supernatant were transferred to a new tube and 500 µl Chloroform:Isoamyl alcohol 24:1 were added. After mixing, the samples were centrifuged for 15 min at $16.000 \times g$ and 500 µl of the clear watery phase were transferred into to a new tube prefilled with 500 µl 2-propanol (pre-cooled). The mixture was incubated for 30 min at room

temperature and subsequently centrifuged for 15 min at $16.000 \times g$. The clear supernatant was discarded and 500 µl ethanol (70%) were added. A centrifugation step at $16.000 \times g$ for 5 min was performed and the clear supernatant was discarded. The pellet was dried at 50 °C and dissolved in 100 µl 0.2 $\times$ TE buffer (1 $\times$ TE: 10 mM Tris-HCl, pH = 9.0).

Extracted DNA was further purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.4. Fluorimetric and photometric DNA analysis

DNA purity was analysed by obtaining ratios at 260/280 nm and 260/230 nm using a Nanodrop UV-vis Spectrophotometer (Thermo Scientific, Wilmington, USA). The Quant-iT PicoGreen dsDNA reagent (Life Technologies, Darmstadt, Germany) and a GENios Plus microplate reader (Tecan, Männedorf, Switzerland) were applied according to the manufacturer's instructions to assess the DNA quantity.

2.5. Real-time PCR analysis

Real-time PCR (qPCR) assays were performed in a total volume of 25 µl using the ABI 7900HT system (Life Technologies GmbH, Darmstadt, Germany), amplifying a small fragment of the *actin* reference gene. The qPCR master mix consisted of 5 µl template DNA, primers and hydrolysis probe (forward: CAA GCA GCA TGA AGA TCA AGG T, 0.9 µM; reverse: CAC ATC TGT TGG AAA GTG CTG AG, 0.9 µM; probe: FAM-CCT CCA ATC CAG ACA CTG TAC TTY CTC TC-TAMRA, 0.2 µM; (Laube et al., 2012); synthesised by Life Technologies GmbH, Darmstadt, Germany, HPLC purified), 1 $\times$ GoTaq® Probe qPCR Master Mix and 0.3 µM CXR reference dye (Promega GmbH, Mannheim, Germany). The following cycling conditions were used: 95 °C for 2 min; 45 cycles of 95 °C for 15 s and 60 °C for 1 min. DNA isolated from rapeseed served as a positive control, whereas water was used as a NTC. Samples were analysed in duplicates and different dilutions (undiluted, 1/4 and 1/16) were used to check for inhibitory effects. Possible storing effects were determined by re-analysing extracted samples (Maxwell and CTAB) after 11 months of storage at -20 °C.

Data are presented as $Cq \pm SD$ (Cq = cycle of quantification; SD = standard deviation (Bustin et al., 2009)).

2.6. Statistics

Results obtained by using the two different DNA extraction protocols were checked for normal distribution, using the Shapiro-Wilk test, and compared by applying the *t*-test or the Mann-Whitney Rank Sum test. *P*-values < 0.05 are considered statistically significant.

2.7. Routine samples

DNA from twelve honey samples obtained on different local markets was isolated using the optimised automated DNA extraction procedure and the commonly used manual CTAB buffer-based DNA extraction method. Isolated DNA was quantified using the PicoGreen reagent. Purity was checked using the Nanodrop Spectrophotometer. Afterwards, all samples underwent qPCR analysis by amplifying a small fragment of the *actin* reference gene. Each sample was analysed in duplicates. Data are presented as $Cq \pm SD$.

3. Results

3.1. Workflow of the automated DNA extraction

The optimisation process led to the following automated DNA extraction protocol: four 50 ml Falcon tubes are filled with 12.5 g

honey. Additionally, 45 ml H₂O are added to each tube. The honey is dissolved by shaking the tubes, followed by a centrifugation step for 15 min at 4.000 × g. The supernatant is discarded and the pellet is solved in 5 ml H₂O. Subsequently, all 4 tubes for each sample are pooled and filled up with H₂O to a total volume of 30 ml. After centrifugation for 10 min at 4.000 × g, the clear supernatant is discarded and the pellet is resuspended in 500 µl CTAB extraction buffer and 20 µl Thioglycerol (THG; Promega GmbH, Mannheim, Germany). This solution is transferred to a 2 ml tube pre-filled with zirconium beads and homogenised in a swing mill with a frequency of 20/sec. 10 µl RNase A (10 mg ml⁻¹) and 40 µl Proteinase K (20 mg ml⁻¹) are added. After incubation for 90 min. at 65 °C, samples are centrifuged at 16,000 × g for 10 min. The clear supernatant is transferred to the first well of a Maxwell® 16 FFS Nucleic Acid Extraction System cartridge. 300 µl Lysis buffer (included in the kit) are added and plungers are placed in the respective well of the cartridge. Before starting the run, 110 µl elution buffer (included in the kit) are filled into the elution tubes, which are placed at the respective position in the Maxwell rack. The rack is inserted into the Maxwell 16 instrument and the run is started by selecting RUN ► DNA ► BLOOD. Samples should be stored appropriately after the run (≈ 43 min). The whole workflow is illustrated in Fig. 1.

3.2. Fluorimetric and photometric DNA analysis

DNA isolated either by using the Maxwell instrument or a manual CTAB buffer-based DNA extraction underwent fluorimetric and photometric analysis. PicoGreen measurements revealed a statistically significant higher DNA yield after automated DNA extraction (Maxwell®: 10.7 ± 1.5 ng µl⁻¹; manual DNA extraction: 4.1 ± 0.5 ng µl⁻¹). Either of the extraction methods resulted in 260/280 nm ratios of 2.0 (Fig. 2).

The automated DNA extraction results in high-grade DNA with a higher yield compared to the manual DNA extraction.

3.3. Real-time PCR analysis

A pooled honey sample was extracted twelve times with each DNA isolation method and analysed in duplicates. Both methods are comparable regarding the Cq values after real-time PCR analysis. For undiluted samples, Cq values of 28.99 ± 0.47 were obtained using the Maxwell extraction robot, whereas the manual CTAB buffer-based DNA extraction resulted in Cq values of 29.04 ± 0.22. These differences are not statistically significant ($P = 0.093$). The automated DNA extraction workflow exhibits good repeatability as a RSD_r (relative repeatability standard deviation) of 1.67% was achieved. Dilutions of 1/4 and 1/16 were analysed to determine potential inhibitory effects. PCR inhibition was not observed, as Cq values of the 1/4 dilutions were 2 units higher compared with undiluted samples after automated DNA extraction (30.88 ± 0.47) or CTAB buffer-based DNA extraction (30.87 ± 0.29). After 1/16 dilution, Cq values were 4 units higher compared with undiluted samples (Maxwell: 32.79 ± 0.44; CTAB: 32.81 ± 0.38), as theoretically expected. The results are depicted in Fig. 3. We can therefore conclude that results of the automated DNA extraction procedure are comparable with the manual CTAB buffer-based DNA extraction protocol.

Regarding the reproducibility of the DNA extraction, we analysed the DNA of one sample extracted on three consecutive days by qPCR. Cq values of 29.59, 30.02 and 30.64 were obtained, which underlines the high reproducibility of the automated DNA extraction procedure.

Storing of the extracted DNA samples for 11 months resulted in a Cq value increase of one for both extraction procedures (manual and automated). Therefore we can conclude that there are no

additional degradation effects after storing samples isolated by our established automated DNA extraction protocol compared to the manual extraction procedure.

3.4. Routine samples

The applicability of the optimised automated DNA extraction procedure was reassessed by analysing twelve routine honey samples using real-time PCR. Cq values were slightly lower or comparable using the Maxwell instrument. Differences in Cq values were not statistically significant. Cq values of the undiluted samples were two units lower than the 1/4 dilution and four units lower than the 1/16 dilution. The results are outlined in Table 1. Therefore, we did not observe a PCR inhibition. Hence, the automated DNA extraction procedure gives slightly lower Cq values ($\Delta Cq \approx 0.5$) results compared to the manual CTAB buffer-based isolation method.

4. Discussion

More and more laboratories are engaged in DNA analysis of honey samples with regard to GMO detection, confirmation of origin of honey, detection of microorganisms or determination of allergenic components. The increasing need for rapid and standardised DNA extraction protocols brought us to the development of an automated DNA extraction. Currently, DNA is mostly isolated by using a CTAB-buffer based DNA extraction, which is laborious and time-consuming. By altering extraction conditions and several components of the kit chemistry, we were able to combine the chemistry of the kit with a CTAB-buffer based DNA extraction. Thereby, we could affiliate the thorough DNA extraction capacity of the CTAB buffer with a highly efficient binding of the DNA to the paramagnetic beads and the removal of inhibitors.

The extraction robot and the cartridges are easy to handle and the DNA extraction workflow is fast and uncomplicated. Hence, it is easy to implement this method in the laboratory. The use of toxic or harmful substances is minimised to CTAB and thioglycerol. The overall time from sample to isolated DNA is reduced from 1.5 d (CTAB) to 4 h (Maxwell). Due to high degree of automation, this new method also reduces the hands-on time in the laboratory. One should keep in mind, that the manual CTAB extraction protocol includes an overnight incubation step. This step is not needed using the automated DNA extraction method. Variances caused by personal bias in pipetting and the possibility of cross-contamination are minimised. This is supported by (Liu et al., 2012) who extracted RNA from influenza virus using the Maxwell extraction robot. The possibility of cross-contamination when using viruses as a sample is much higher compared to honey as a sample matrix. The authors also state that the use of paramagnetic beads and disposable equipment reduces the likeliness of cross contamination.

DNA yields after automated DNA extraction were higher compared to the manual DNA extraction. DNA concentrations measured after manual CTAB-buffer based extraction were lower as reported by Waiblinger et al. (2012), because we used the intercalating PicoGreen reagent for DNA quantification. The photometric DNA quantification by A₂₆₀ measurement overestimates the DNA quantity. In-house validation revealed a high repeatability and equivalent real-time PCR results compared to the manual isolation method. No PCR inhibition was observed which underlines the effective purification of the DNA and removal of potential inhibitors. There are no additional adverse effects on DNA when stored at -20 °C compared to the manual extraction.

The real-time PCR Master Mix used for DNA analysis is of high importance. First, we used the TaqMan® Universal PCR Master Mix (Life Technologies GmbH, Darmstadt, Germany), which resulted in

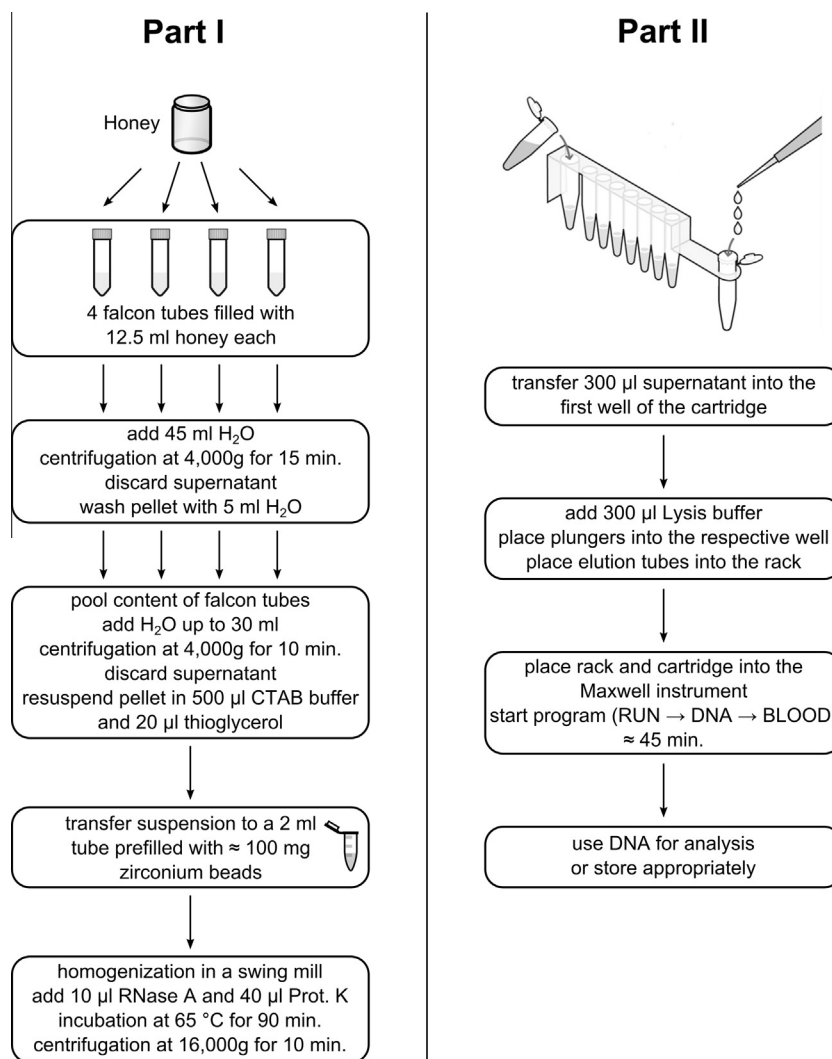


Fig. 1. Schematic workflow of the automated DNA extraction from honey.

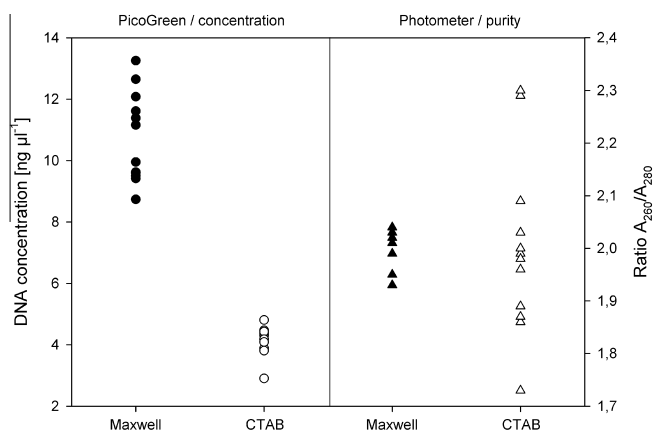


Fig. 2. DNA concentration and purity after automated (Maxwell) or manual (CTAB) DNA extraction from the pooled honey sample; left y-axis for PicoGreen results and right y-axis for 260/280 nm ratio.

severe PCR inhibition after manual DNA extraction and moderate PCR inhibition after automated DNA extraction (data not shown). As a consequence, we switched to the GoTaq[®] Probe qPCR Master Mix by Promega GmbH, which seems to cope much better with

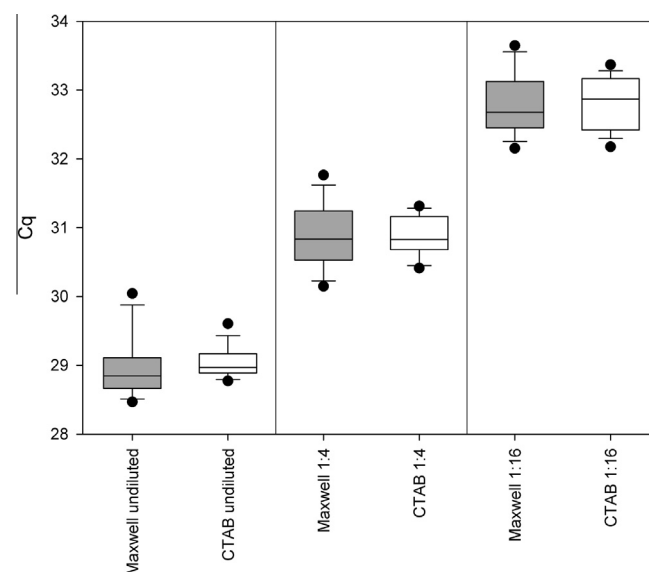


Fig. 3. qPCR results obtained after automated (Maxwell) or manual (CTAB) DNA extraction from a pooled honey sample; Cq = cycle of quantification.

Table 1
qPCR results for 12 different routine honey samples after optimised automated DNA extraction in comparison to results after a commonly used manual CTAB buffer-based DNA extraction (Cq = cycle of quantification, SD = standard deviation).

Sample	Maxwell undiluted (Cq _{mean} ± SD)	CTAB undiluted (Cq _{mean} ± SD)	Maxwell 1:4 diluted (Cq _{mean} ± SD)	CTAB 1:4 diluted (Cq _{mean} ± SD)	Maxwell 1:16 diluted (Cq _{mean} ± SD)	CTAB 1:16 diluted (Cq _{mean} ± SD)
Honey 1 multi-floral honey	30.35 ± 0.10	31.34 ± 0.23	32.00 ± 0.08	33.08 ± 0.25	34.23 ± 0.16	34.86 ± 0.05
Honey 2 forest honey	26.79 ± 0.01	27.77 ± 0.04	28.64 ± 0.11	29.82 ± 0.05	30.36 ± 0.13	31.62 ± 0.05
Honey 3 multi-floral honey	26.79 ± 0.01	28.65 ± 0.03	28.64 ± 0.11	30.46 ± 0.10	30.36 ± 0.13	32.75 ± 0.17
Honey 4 multi-floral honey	30.14 ± 0.01	30.86 ± 0.06	31.71 ± 0.16	32.69 ± 0.35	33.87 ± 0.57	34.26 ± 0.06
Honey 5 rosemary honey	28.81 ± 0.18	29.22 ± 0.24	30.99 ± 0.21	31.51 ± 0.05	32.71 ± 0.15	33.36 ± 0.10
Honey 6 multi-floral honey	32.00 ± 0.05	32.20 ± 0.17	34.13 ± 0.32	34.18 ± 0.01	36.54 ± 0.49	36.66 ± 0.22
Honey 7 multi-floral honey	28.77 ± 0.09	29.89 ± 0.04	30.85 ± 0.07	31.50 ± 0.07	32.85 ± 0.28	33.54 ± 0.33
Honey 8 multi-floral honey	26.20 ± 0.20	26.39 ± 0.29	28.01 ± 0.24	28.06 ± 0.24	29.91 ± 0.32	30.08 ± 0.01
Honey 9 eucalyptus honey	26.34 ± 0.11	27.26 ± 0.09	28.38 ± 0.15	29.49 ± 0.10	30.70 ± 0.18	31.55 ± 0.23
Honey 10 multi- floral honey	30.03 ± 0.44	30.39 ± 0.28	31.99 ± 0.12	32.58 ± 0.29	34.11 ± 0.41	34.50 ± 0.30
Honey 11 multi- floral honey	30.56 ± 0.09	28.69 ± 0.21	32.12 ± 0.31	30.13 ± 0.12	33.77 ± 0.12	32.74 ± 0.10
Honey 12 multi- floral honey	31.50 ± 0.04	31.63 ± 0.36	33.31 ± 0.37	33.69 ± 0.13	35.68 ± 0.23	35.68 ± 0.04

residual inhibitory effects than the TaqMan® Universal PCR Master Mix.

The applicability of this method was further demonstrated by DNA extraction from different honey samples. The automated DNA extraction revealed equal or slightly lower Cq values for all tested honey samples compared to the manual extraction method. The automated extraction method is therefore suitable for a broad range of honey samples from various origins.

As a conclusion, we combined a thorough CTAB buffer-based DNA extraction with the efficient DNA purification by using the Maxwell® 16 instrument. This method can be used for any application dealing with DNA analysis of pollen in honey.

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