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ANALYSIS OF DIFFERENT TYPES OF HONEY TO EMPHASIZE GENETICALLY MODIFIED SEQUENCES

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Abstract: Food safety and consumer health protection is a particularly important aspect, always in the attention of researchers. The authentication of honey, but also its mislabelling, is one of the current challenges related to quality monitoring. Therefore, a rigorous monitoring of the different types of honey on the market is required, to ascertain whether they are properly labeled and to detect the possible presence of modified genes. The aim of this study was to analyze honey samples, to identify the possible presence of genetically modified genes, using the technique based on DNA analysis, called real-time PCR. For this purpose, ten samples of honey were analyzed. DNA isolation was performed with Quick-DNA™ Plant/Seed Miniprep kit. For identification a potential presence of the genetically modified genes in the honey samples it was used a real-time PCR kit - Xpert qDetect P-35S, T-NOS and P-FMV -DNA amplification kit, that allows detection by real-time PCR of specific DNA sequences from the 35S promoter, NOS terminator and/or FMV promoter present in total DNA previously purified from honey samples. The results showed that two honey samples - M2 and M3 contain genetically modified sequences. It can be concluded that these results illustrate the importance of honey quality monitoring for the effective detection of genetically modified organisms.

Keywords: honey, genetically modified sequences, food quality, screening, real-time PCR

1. Introduction

Honey is a natural product produced by honeybees (*Apis mellifera* L.) from various plant secretions (Bertelli et al., 2010; vanEngelsdorp and Meixner, 2010; Siddiqui et al., 2017). Honey is marketed as a food with scientifically and clinically proven content to have beneficial health properties. Honey has many health benefits arising from the regular consumption of honey, such as the elimination of disorders of the gastrointestinal tract and cardiovascular disorders of the cardiovascular system (vanEngelsdorp and Meixner, 2010; Majtan et al., 2021). Some studies showed an antioxidant activity of honey (Jamróz et al., 2014; Piljac-Zegarac et al., 2009) and showed that there was a direct correlation between the content of phenols, the antioxidant activity and the color intensity of the honey. Due to the presence of phenolic constituents, honey has antibacterial activity (Majtan et al., 2021) anticancer, anti-inflammatory (Samarghandian et al., 2017), antithrombotic, immune, immunomodulatory and analgesic properties. Some studies have also shown that honey decreases cardiovascular risk factors without an

increase in body weight (Yaghoobi et al., 2008; Siddiqui et al., 2017).

Genetically modified organisms (GMOs) are organisms in which the genetic material is modified, by introducing portions of DNA from another organism, to give it resistance or to increase its production. Thus, new crops were obtained, resistant to pests, herbicides and insecticides, and the production of many plants such as: corn, rapeseed, potato, rice, sugar beet was increased. After 1998 there was an exponential increase in genetically modified foods. Among the plants that have had a rise and a special economic importance are: corn, cotton, soybeans, tomatoes, potatoes, rapeseed, flax and many others (Cristea and Denaeyer, 2004).

According to DIN ISO 9000, the term "quality" is defined as ”the totality of characteristics relevant to the ability of a product to fulfill its requirements”. In order to obtain healthy and safe products, without neglecting economic and ecological aspects, the concept of food quality should be much broader. Thus, several aspects must be taken into account: requirements of the producer, consumer, supervision and legislative bodies (Böhme et al., 2016; Müller and Steinhart, 2007).

As defined by European Directive 18/2001/EC, in 2018, the European Court of Justice (ECJ) showed that organisms obtained through mutagenesis are also genetically modified organisms (GMOs) (Broll et al., 2019; Kuntz, 2020; Ryan et al., 2020). Genetically modified (GM) pollen not authorized in the EU cannot be present in honey. The honey must be labeled if it contains more than 0.9% pollen from authorized GM plants in relation to the total pollen content (Zmijewska et al., 2013; Villanueva-Gutierrez et al., 2014).

Food adulteration as well as it’s mislabeling has become a cause of concern for people worldwide. To monitor and control the authenticity of food, but also to guarantee the correct and accurate labeling of food products, the use of analytical methods is necessary. Thus, using these methods, we must ensure that the components included in a food product are of the nature and quality declared by the seller (Cheftel, 2005; Ortea et al., 2016).

Traditional methods such as SDS-PAGE and other classical genetic techniques such as restriction fragment length polymorphism (RFLP), real-time polymerase chain reaction (Real-Time PCR) are still used based on molecular masses. Also, methods such as techniques related to proteomics, metabolomics and genomics are increasingly used, helping to elucidate the limitations of previous methodologies (Böhme et al., 2016; Ortea et al., 2016).

DNA-based techniques are considered routine analyzes for food analysis. They enable the detection of food fraud in complex foods. Many of the classical polymerase chain reaction (PCR)-based techniques (DNA sequencing, RFLP, multiplex-PCR, real-time PCR, microarray, random amplified polymorphic DNA, and microsatellites) have been used to determine species, cultivars, and geographic origin in food products. At the same time, new techniques based on genomics were used to verify the authenticity of food products. Thus, attempts have been made to improve the performance of classical DNA-based techniques in terms of specificity, sensitivity and sample processing capacity (Böhme et al., 2016; Haynes et al., 2019).

The aim of this study was to analyze honey samples, to identify the possible presence of genetically modified genes, using the technique based on DNA analysis, called real-time PCR.

2. Materials and methods

For the DNA-based technique, 10 honey samples from different market were analyzed in the study, coded as follows: M1-M10.

DNA isolation from honey samples was carried out using the Quick-DNATM Plant/Seed Miniprep kit - Zymo Research, which provides a simple and rapid isolation of high-quality DNA for PCR analysis, without inhibitors, from a variety of plant sample sources (Instruction manual - Quick-DNA^{TM} Plant/Seed Miniprep kit).

The amplification of DNA extracted from honey samples was carried out with the help of the Xpert qDetect P-35S, T-NOS and P-FMV kit (Grisp Research Solutions), which allows the detection by Real-Time PCR of specific DNA sequences from the 35S promoter, NOS terminator and/or FMV promoter present in total DNA previously purified from honey samples. The detection limit is 10-100 µg of GMO DNA, which allows detection of only 0.01-0.1% of target DNA in food samples if 100 ng of total DNA is used (Instruction manual - Xpert qDetect P-35S, T-NOS and P-FMV). The parameters of the amplification program are detailed in **Table 1**.

Table 1. The parameters of the amplification program (Instruction manual - Xpert qDetect P-35S, T-NOS and P-FMV)

Steps	**Temperature**	**Time**	**Cycles number**
Enzyme activation	50˚C	2 minutes	1
Initial Denaturation	95˚C	5 minutes	1
Denaturation	95˚C	30 seconds	40
Primer alignment	60˚C	30 seconds + channel data acquisition FAM and ROX	
Extension	72˚C	30 seconds	

3. Results

Since 1994, more than 100 genetically modified plants have been approved for use as food or animal feed. Effective detection of genetically modified organisms in food and feed is essential for the implementation of national legislation. Given the high diversity, an initial generic screening for the presence of the most common genetically modified materials is usually the first step in GMO analysis to reduce the volume of subsequent identification analyses. Since the 35S promoter from cauliflower mosaic virus (CaMV) and the NOS terminator from *Agrobacterium tumefaciens* are the most common elements present in transgenic materials in food and feed, detection of these regulatory sequences by PCR amplification is the most logical choice. However, as they do not cover some important GMOs, such as soybean MON89788, sugar beet H7-1 or rapeseed GT73, to ensure the widest possible detection, the kit used is also suitable for promoter detection from FigWort mosaic virus (P-FMV) by PCR (Instruction manual - Xpert qDetect P-35S, T-NOS and P-FMV).

Specific amplified DNA target sequences could be observed in real-time in the amplifier software. Each amplification curve recorded represents relative fluorescence units for each individual sample compared to a fluorescence threshold value, depending on the fluorophores used in the reaction (FAM and ROX channels). The analyzed samples are considered to be

positive, if a fluorescence value higher than the fluorescence threshold value is recorded. At the same time, the amplifier software automatically records the Ct (Threshold Cycle) values, values that are detected, for each individual sample, at the intersection between the amplification curve and the fluorescence threshold signal value. For each sample, 2 analyzes were performed in repetition, the device automatically calculating the average of the analysis values for the 2 analyses.

According to the specifications of the Xpert qDetect P-35S, T-NOS and P-FMV kit (Grisp Research Solutions), a sample is considered positive if a positive Ct is recorded for both FAM and ROX channels or if the Ct is positive for FAM channel and Ct = N/A on ROX channel. Samples are considered negative if Ct = N/A on the FAM channel and Ct is positive on the ROX channel. If both the GMO channel (FAM channel) and the internal control (ROX channel) have signals below the fluorescence threshold level (Ct = N/A), it means that the PCR reaction has been inhibited. For positive control samples, they must be positive for the FAM channel and positive but not significant for the ROX channel. Negative control samples should record Ct = N/A for both channels.

The results recorded for the FAM channel show whether the sample is positive for the presence of GMOs, and the results recorded for the ROX channel show whether the amplification reaction proceeded under normal conditions without being inhibited. The results showed that the positive control samples recorded positive Ct values for the FAM channel, and for the negative control samples, the Ct values were below the fluorescence threshold level. All amplification reactions showed detectable results.

Honey samples M2 (Ct FAM = 28.22; Ct ROX = 23.33) and M3 (Ct FAM = 35.11; Ct ROX = 23.81) were detected as positive only for the P-FMV sequence, recording positive Ct values for both detection channels. In the case of the other honey samples, the recorded results were negative for all 3 analyzed sequences (P-35S, T-NOS, P-FMV). These results show that honey samples M2 and M3 contain genetically modified sequences (**Table 2**).

Table 2. Amplification results for all samples analyzed

Sample type	**Ct values**					
	P-35S		**T-NOS**		**P-FMV**	
	FAM	**ROX**	**FAM**	**ROX**	**FAM**	**ROX**
M1	N/A	24.66	N/A	23.28	N/A	24.12
M2	N/A	24.20	N/A	23.46	28.22	23.33
M3	N/A	24.55	N/A	24.18	35.11	23.81
M4	N/A	24.26	N/A	24.23	N/A	24.06
M5	N/A	24.25	N/A	24.00	N/A	24.55
M6	N/A	24.05	N/A	24.37	N/A	24.62
M7	N/A	23.85	N/A	24.22	N/A	23.93
M8	N/A	24.11	N/A	24.82	N/A	24.41
M9	N/A	24.87	N/A	24.85	N/A	24.43
M10	N/A	24.76	N/A	24.87	N/A	24.46
Pozitive control	25.23	23.62	24.45	23.50	24.34	23.16
Negative control	N/A	N/A	N/A	N/A	N/A	N/A

In a study that carried out the analysis of honey from the Yucatan peninsula in Mexico, the authors showed that following the PCR analysis, 2 positive samples for genetically modified soy out of a total of 9 samples were confirmed, which demonstrates the importance of monitoring the quality of honey (Villanueva-Gutierrez et al., 2014). Another study describes the possibilities and limits of detection of genetically modified rape in rape honey by real-time PCR and the sensitivity of PCR methods for the detection of genetically modified rape was demonstrated (Waiblinger HU et al., 2005).

Since cotton is one of the main plants used as a source of honey, Chinese researchers became concerned to see if there are genetically modified DNA sequences contained in honey. Researchers have developed a DNA extraction procedure and a PCR protocol for the detection of foreign DNA sequences in bee honey. They used a PCR protocol, whereby genetically modified cotton DNA sequences, 125-550 bp in length, could be specifically amplified and detected (Cheng et al., 2007).

The increasing number and diversity of genetically modified organisms for the food and feed market requires the development of advanced methods for their identification and detection. This problem can be solved by using next generation sequencing (NGS) and DNA barcoding techniques (Saltykova et al., 2022).

Conclusions

The results obtained in this study illustrate the importance of honey quality monitoring for the effective detection of genetically modified organisms, but also the effectiveness of the real-time PCR technique for performing an initial screening to detect the presence of genetically modified sequences. Taking into account the results obtained, a complete analysis is required, to quantify the genetically modified sequences, but also to verify the authenticity of all the ingredients in the honey samples, using other methods (DNA barcoding, next generation sequencing).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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