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Detection of honey adulteration by conventional and real-time PCR

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ABSTRACT

This work applies both conventional and real-time PCR DNA amplification techniques for detecting and quantifying rice molasses in honey. Different levels of adulteration were simulated (1, 2, 5, 10, 20, 50%) using commercial rice molasses. Among the different specific genes of rice tested by PCR, the PLD1 primer was the most effective. This allowed the visualization in agarose gel of this type of adulterant up to 5-20%. Moreover, by means of real-time PCR it was possible to distinguish the different levels of rice DNA, and therefore the percentage of adulteration (1-50%). A standard curve built with the DNA serial dilutions of rice genomic DNA concentrations showed that the quantification level was between 2-5%. These results offer compelling evidence that DNA techniques could be useful not only for the detection of adulterations of honey with rice molasses but also for the quantification of levels lower than those of conventional techniques.

Keywords

Honey authentication; conventional-PCR; real-time-PCR.

1. Introduction

Honey is a natural sweet substance that no alterations are permitted. This means the addition of substances, as well as the elimination of pollen or any intrinsic component is prohibited (Council Directive, 2002, Real Decreto 1049/2003). Honey is highly vulnerable to food fraud which accounts for approximately 90% of all adulterations related to sweeteners (Sobrino-Gregorio, Vargas, Chiralt & Escriche, 2017). Guaranteeing the purity in honey is a priority for producers and regulatory authorities in addition to avoiding economic fraud and ensuring public health. As a result, controlling this aspect of the quality in honey has become increasingly important (Cai et al., 2013; Sobrino-Gregorio, 2017).

Generally, honey is adulterated with other cheaper sweeteners such as sugar syrups, which could have a similar sugar composition (Cai et al., 2013). The most common adulteration is with rice syrups or rice molasses, used in some Asian countries, where most of the honey is exported to Europe, the USA and Japan (Sobrino-Gregorio et al., 2017). As a result, the European Commission is promoting the development of simple analytical methods that permit the detection of adulterated honey (Council Directive, 2002).

In recent years, a number of these methods have been used to differentiate genuine honey from adulterated ones (Ulberth, 2016; Siddiqui, Musharraf, Choudhary & Rahman, 2017). Among them, the most used by the analytical laboratories focusing on quality control of honey are: NMR spectroscopy

(although it is the most recognized, it is very expensive and time-consuming requiring a data library to compare the results) (Bertelli et al., 2010; De Oliveira et al, 2014), and enzymatic activity (diastase, invertase) (Serra, Soliva & Muntane, 2000), among others. The drawback to using only one of these techniques is that results are not always conclusive. Therefore, it is necessary to use more than just one to achieve a reliable report. Furthermore, it slows down the analytical process making it very expensive (Sobrino-Gregorio et al., 2017).

With the aim of analyzing adulterations in honey, other analytical techniques have been recently reported by different authors: Fourier transformation and Raman spectroscopy (to detect the presence of inverted beet and cane syrups) (Oroian & Ropciuc, 2017), differential scanning calorimetry (DSC) (Sobrino-Gregorio et al., 2017), high performance liquid chromatography (HPLC) to detect starch syrups (Wang et al., 2015) and stable carbon isotope mass spectrometry (SCIRA) (Elflein & Raezke, 2008), among others. As with the techniques mentioned above these recent methodologies, alone, have not given conclusive results either.

Among the most promising techniques currently available for the determination of the quality and adulteration of food products, DNA-based methods are of increasing importance (Lo & Shaw, 2018; Al-Kahtani, Ismail & Ahmed, 2017; Meira et al., 2017). The conventional polymerase chain reaction (PCR) (for identification) and real-time PCR (for quantification) techniques, offer results of high specificity and sensitivity, reproducibility, low levels of cross-contamination and reduce analysis time (Meira et al., 2017). These methodologies have been successfully applied for the authentication of animal products like milk (Mayer,

2005), meat (Farrokhi & Jafari Joozani, 2011; Rodríguez-Ramírez, González-Córdova & Vallejo-Cordoba, 2011; Cai, Gu, Scalan, Ramatlapeng & Lively, 2012; Kesmen, Yetiman, Sahin & Yetim, 2012; Safdar, Junejo, Arman & Abasiyanik, 2014; Chen, Wei, Chen, Zhao & Yang, 2015) and seafood (Nebola, Borilova & Kasalova, 2010; Rasmussen, Morrissey & Walsh, 2010; Rodríguez-Ramírez et al., 2011; Fernandes, Costa, Oliveira & Mafra, 2017). Specifically, in honey, this technique has only been used for the botanical origin identification (Laube et al., 2010; Guertle, Eicheldinger, Muschler, Goerlich & Busch, 2014; Soares, Amaral, Oliveira & Mafra, 2015).

Regarding the positive results obtained in the detection of adulterations in products of animal origin, it could be considered viable that this technique can be applied to other animal by-products like honey. However, based on our current knowledge, this method has not been used for the identification of adulteration in honey. With this aim in mind, this study evaluated the capacity of conventional PCR and real-time PCR to identify and quantify the presence of rice molasses in honey samples simulating different levels of adulteration. To achieve this, a previous step was necessary to solve the difficulty of extraction and amplification of rice molasses DNA in honey.

2. Materials and methods

2.1. Sample preparation

Orange blossom honey (*Citrus spp.*), provided by the company "Melazahar" (Montroy, Valencia, Spain), for this study was used. The botanical categorization was performed by means of pollen analysis, which was quantified following the recommendations of the International Commission for Bee Botany (Von Der Ohe, Persano, Piana, Morlot & Martín, 2004). Different

types of rice molasses were used as an adulterant: “Danival” (France) and “Cal Valls” (Spain), respectively codified as I and II.

The samples evaluated in the present work were: pure rice molasses, pure orange blossom honey and mixture of both in different percentages (1, 2, 5, 10, 20 and 50% of rice molasses, respectively) simulating the adulteration of honey. To this end, a 10 g sample with 45 mL of water was incubated at 65 °C with shaking for 30 min approximately, until the sample was completely dissolved (NucleoSpin-Food-isolation of genomic DNA from honey or pollen, 2018).

2.2. Genomic DNA extraction

Different protocols for extracting DNA were tested: the CTAB method (Doyle & Doyle, 1990), the modified CTAB method (Aljanabi, Forguet & Dookun, 1999) and the commercial kit “NucleoSpin Food” (Macherey-Nagel, Germany). The latter, according to the manufacturer's instructions and the additional protocol (NucleoSpin-Food-isolation of genomic DNA from honey or pollen, 2018).

2.3. Rice primers

Three rice primers targeting two different rice-specific genes, used by Takabatake et al. (2015), were considered in this study to achieve sufficient DNA of suitable quality (Table 1). The specificity of the primers was demonstrated in silico comparing the primer sequences against the “nr database” using BLASTn program.

2.4. Conventional PCR

Polymerase chain reaction (PCR) amplifications were carried out on a total reaction volume of 20 µL, containing 1 µL of extracted DNA. The reaction mixture contained 6.8 µL water (Roche, Germany), 10 µL of PCR buffer with

deoxynucleotide triphosphates (dNTPs) (2x) 1.5 mM Mg at 1x, 1.2 µL of $MgCl_2$ 25 mM, 0.2 µL of Taq DNA polymerase 2.5 U/µL (Kapa3g Plant, Kapabiosystems, South Africa) and 0.4 µL of each primer (10 µM). In the reaction, a positive control (rice DNA extracted from a development plant of commercial variety of ssp. *japonica*) and a negative control (water) were included.

PCR was performed using the Thermal Cycler Mastercycler (Eppendorf, Germany) using the following conditions: 95°C/2 minutes followed by 30 cycles of 95°C/15 seconds, 60°C/15 seconds, 72°C/15 seconds, and a final extension at 72°C/10 minutes.

2.5. Agarose gel electrophoresis

The PCR products were separated using electrophoresis with a 3% agarose gel (Conda, Spain). The results were seen under UV light (transilluminator Universal Hood II (Bio-rad), USA). PCR band size was verified with a 100 bp molecular weight marker (FastGene 100 bp DNA Ladder, Genetics, NIPPON Genetics EUROPE GmbH).

2.6. Real-time PCR

The real-time polymerase chain reaction (real-time PCR) amplifications were carried out in a total reaction volume of 10 µL, containing 3 µL of DNA extract. The reaction mixture contained 1.9 µL water (Roche, Germany), 5 µL of master mix 2x Sybr Fast Universal (Kapabiosystems, South Africa) and 0.05 µL of each primer (10 µM). In the reaction, a positive control (rice DNA extracted from a development plant of commercial variety of ssp. *japonica*) and a negative control (water) were included.

Real-time PCR was performed using the real-time PCR LightCycler480 (Roche, Switzerland) with the following conditions: 95°C/10 minutes followed by 45 cycles of 95°C/10 seconds, 65°C/15 seconds, 72°C/15 seconds. Finally, a melting curve was performed by heating 95°C/1 minute, cooling down 40°C/1 minute, and heating again from 60°C to 95°C, performing 25 acquisitions per 1 C.

2.7. Rice DNA concentrations

Serial dilutions of rice genomic DNA (100.00, 50.00, 25.00, 12.50, 6.25, 3.13, 1.56 and 0.78 ng/µL) were amplified by real-time PCR to build the standard curve required to determine the DNA concentration in the samples.

All experiments (conventional PCR, real time PCR and the DNA concentration curve) were carried out at least 4 times.

3. Results and discussion

3.1 Optimization of DNA extraction

The complexity of honey and the highly processed molasses influences the low amounts available of target DNA of these products (Dyshlyuk, Golubtsova, Novoselov & Shevyakova, 2014; Soares et al., 2015). Therefore, the first obstacle to overcome was to have access to sufficient quantity and quality of target DNA that is a necessary condition to be amplified by the PCR later.

With the conventional protocols, CTAB and the modified CTAB, the results were unsatisfactory since no DNA from molasses could be amplified. In consequence the CTAB-based methods were discarded. Only the commercial kit “NucleoSpin Food” provided high quantity and quality DNA extracts, and consequently was selected. In this respect, in other processed food matrixes, the chaotropic solid-

phase extraction “NucleoSpin Food” kit has proved more efficient than CTAB protocols (Garino et al., 2017).

3.2. Conventional PCR for pure rice molasses

The agarose gel images of PCR products, obtained from conventional PCR reactions, using three species-specific primers for rice detection (SPS2, PLD1 and PLD2) in pure rice molasses (I and II), are shown in Figure 1.A and 1.B.

In Figure 1.A the lines represent the PCR products that use rice primers (SPS2, PLD1 and PLD2) for pure molasses I. In this figure, the pure molasses I with PLD1 primers (line 4) had a visible amplification, with a strong and defined band, similar to the positive control with these primers (line 6). The pure molasses I with SPS2 primers (line 1) also resulted in a visible but less intense amplification. The same occurs with its corresponding positive control (line 3). Molasses I with PLD2 primers (lines 7) and the positive control with these primers (line 9), do not show the expected result since their amplifications were very diffused and weak, probably due to the degradation caused by heat and filtration during the elaboration of the molasses (Caldwell, 2017; Mano et al., 2017). In all three cases, the negative control was as it did developing visible amplifications (lines 2, 5 and 8).

Lines of Figure 1.B represent the PCR products that use rice primers (SPS2, PLD1 and PLD2) for pure molasses II. In this figure, the results are very similar for the three types of primers. Molasses II (lines 10, 13 and 16, respectively) showed amplifications with the three types of primers, but always less intense than the positive control (lines 12, 15 and 18, respectively). Again, by not obtaining any amplification implies the negative control was correct (lines 11, 14 and 17).

Considering these results, the two best primers were SPS2 and PLD1 since they provided the best amplification results, producing clear bands of both pure molasses (I and II). For this reason, these primers were chosen for the subsequent experiments.

3.3. Conventional PCR for honey, rice molasses and rice molasses mixtures

Figure 2.A and 2.B shows the agarose gel images of PCR products, obtained from conventional PCR reactions, using two species-specific primers for rice detection (SPS2 and PLD1), in pure rice molasses I, pure orange blossom honey and mixture of both in different percentages (1, 2, 5, 10, 20 and 50%, respectively) simulating the adulteration of honey.

Lines of Figure 2.A and 2.B, respectively, represent PCR products for pure molasses I that use SPS2 and PLD1 rice primers. The absence of a visible amplification in the honey sample (lines 1 for SPS2 and 9 for PLD1), and in the negative control (C-), demonstrates the absence of rice DNA. This is a clear indication that this honey has not been adulterated with this type of molasses. Furthermore, it is observed that with the addition of 1% and 2% of molasses (lines 2, 3 for SPS2 and 10, 11 for PLD1) amplification bands are visible, but they are very faint. On the contrary, from 5% to 50% of molasses (lines 4-7 for SPS2 and lines 12-15 for PLD1) there are definite amplifications that increase in intensity. Finally, the pure molasses I (lines 8 for SPS2 and 16 for PLD1) can be found with the most intense band next to the positive control band (C+). Summarizing, the same results were obtained for both primers, although PLD1 showed the most intense amplification.

On the other hand, Figure 3.A and 3.B displays the agarose gel images of PCR products, obtained from conventional PCR reactions, using two species-specific

primers for rice detection (SPS2 and PLD1) in pure rice molasses II, pure orange blossom honey, and mixture of both in different percentages (1, 2, 5, 10, 20 and 50%, respectively) simulating the adulteration of honey.

Lines of Figure 3.A and 3.B, respectively, represent PCR products for pure molasses II using SPS2 and PLD1 rice primers. In this case, up to 10% adulteration (lines 5 for SPS2 and 13 for PLD1) does not produce a visible amplification. For adulteration between 10% and 20%, the bands are very weak (lines 5, 6 for SPS2 and 13, 14 for PLD1) and more defined amplifications appearing for 50% and for pure molasses II (lines 7, 8 for SPS2 and 15, 16 for PLD1). In both cases (Figure 3.A and 3.B) something similar occurs, although in Figure 3.B (PLD1 primers) the amplification for 10% and 20% are better appreciated. The differences among both molasses in the amplification results obtained is a possible consequence of the variations in the heating and filtering processes used for obtaining them, which may affect DNA integrity (Caldwell, 2017; Mano et al., 2017).

These conventional PCR experiments were repeated at least 4 times obtaining the same banding pattern, which indicates the reproducibility of the results and the integrity of the DNA samples. In all cases the controls (C+ and C-) verified the results obtained.

3.4. Real time PCR amplification

Figure 4 shows, as an example, a representative picture of a real-time PCR result for pure honey (H) and honey with different percentages of pure rice molasses (I and II), simulating the same levels of adulteration as in conventional PCR. All levels of adulteration can be appreciated in the corresponding order (1,

2, 5, 10, 20 and 50%). However, the adulteration of honey-molasses II with PLD1 primer showed no differences between 1% and 2% (Figure 4.D).

When observing in more detail the Cp values (crossing point-PCR-cycle), the order was altered in some cases. For molasses I and II using primers SPS2 (Figure 4.A and 4.C), the difference between each of the adulteration samples is very small. This causes an incorrect order in their Cp values: 50% (26.14), 20% (27.22), 5% (28.17), 2% (28.49), 10% (28.50), 1% (29.68) and honey (H) (31.33) with molasses I. In relation to molasses II: 50% (28.53), 20% (29.18), 10% (29.22), 1% (29.68), 5% (29.84), 2% (30.20) and honey (H) (30.75).

For molasses I and II using primers PLD1 (Figure 4.B and 4.D) the results are much better. In this case, the order of the levels of adulteration (in both molasses types) is as follows based on their Cp value: 50% (27.45, 29.12), 20% (28.42, 31.13), 10% (29.95, 31.27), 5% (30.68, 32.57), 1% (32.25, 34.65), 2% (32.74, 36.27). Only 1% and 2% are altered, with very little differences between them, however, a clear difference is observed with respect to pure honey (H). Using PLD1 primer increased and ordered values higher than 5% are considered satisfactory.

In all cases, the positive control has the smallest value of Cp, followed by the corresponding rice molasses. In the case of negative controls, it has the Cp value of the highest value with SPS2 (29.62 in Figure 4.A and 34.94 in Figure 4.C) or completely negative, as it appears in the analyses carried out with the PLD1 primer (Figure 4.B and 4.D).

The results demonstrated the specificity and sensitivity of the real-time PCR analyses for rice molasses detection over the conventional PCR (Lubis, Salihah, Hossain & Ahmed, 2017), and more in the case of PLD1. Since these

primers have an amplicon smaller than the rest (68bp), they have the capacity to amplify smaller DNA chains or highly degraded DNA (Wiseman, 2002). It is possible to affirm that combining real-time PCR with PLD1 primer could be considered the perfect screening or semi-quantitative technique for the detection of rice molasses in honey. For this reason, these primers were chosen for the subsequent experiment.

Similar results were obtained in all real time PCR experiments which demonstrated how well the results can be reproduced. In addition, the melting curve analysis showed that there was non-specific amplification in none of the experiment.

3.5. Rice DNA concentrations

To know the concentration of DNA present in the samples a standard curve was built plotting the Cp values against the logarithms of DNA serial dilutions of rice genomic DNA concentrations (100.00, 50.00, 25.00, 12.50, 6.25, 3.13, 1.56 and 0.78 ng/µL) (Figure 5). The regression coefficient of 0.999 highlights the good correlation existing in the range established between Cp values and log concentrations of rice template DNA. Table 2 shows the calculated rice DNA concentrations (from the standard curve) for all the samples evaluated in this study. These values ranged from 0.395 to 0.017 and 0.132 to 0.003 ng/µL rice DNA, for rice molasses I and II, respectively. A progressive and ordered decrease of these concentrations is observed in relation to the lowering of the adulteration level. It can be stated that for both molasses it was not possible to differentiate between 2% (0.012 and 0.001 ng/µL) and 1% (0.017 and 0.003 ng/µL) of adulteration since the values obtained between these percentages are very close. This situation is common in real-time PCR analyses when DNA is

295 highly degraded (Alonso-Rebollo, Ramos-Gómez, Busto & Ortega, 2017).
296 Nevertheless, between 2% and 5%, a clear differentiation is observed, therefore
297 between these both concentrations an acceptable limit of quantification could be
298 established.

299 Considering the difficulty of the studied matrices (honey and molasses), in
300 relation to the low amounts of target DNA, the capability of detecting a level of
301 adulteration around 2-5% is considered an excellent result. Furthermore, it is
302 important to point out that the techniques that are currently established to detect
303 the incorporation of this specific type of molasses in honey is not able to
304 guarantee a detection of adulteration below 10% of adulteration (Xue et al.,
305 2013).

306 Using the same technique as in this study, similar identification adulteration
307 percentages were reported by Al-Kahtani et al. (2017) when detecting pork
308 meat in chicken meat, since pork DNA below 5% adulteration was not detected.
309 Nevertheless, in the case of other types of meat (beef, camel, rabbit, goat and
310 sheep) the same authors detected up to 1% adulteration.

311 **4. Conclusions**

312 This paper has presented for the first time that the PCR technique can be
313 applied to quantify the presence of rice molasses in honey. This novel approach
314 has been introduced to detect this kind of fraud in a bee product in which any
315 type of addition is allowed. It was demonstrated that by using an appropriate
316 genomic DNA extraction, it is possible to overcome the main obstacle in
317 accessing sufficient quantity and quality of target DNA that is a necessary
318 condition, to be amplified by the PCR later. Several specific genes of rice were
319 used by conventional PCR technique, which allows the detection of this type of

adulterant in honey. Furthermore, by means of real-time PCR it was also possible to distinguish the different levels of rice DNA present in mixtures of honey and rice molasses. By means of a standard curve (built with the DNA serial dilutions of rice genomic DNA concentrations) it was possible to quantify the amount of rice DNA and therefore to estimate more accurately the level of adulteration (up to 2-5%). The percentage of quantification achieved by PCR technique implies a better advantage over other more expensive and time-consuming methodologies that are not able to reach a level lower than 10%. However, further clarification is necessary to determine whether these findings could be applied to the detection of other kinds of molasses in honey, since the limiting factor could ~~probably~~ be the DNA extraction corresponding to the species from which the respective molasses are obtained.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Figure Caption

Figure 1. Agarose gel electrophoresis of PCR products for pure molasses (I and II) using rice primers (SPS2, PLD1 and PLD2). Figure 1.A: M: marker; 1-3: pure molasses I, negative and positive control with SPS2 primers; 4-6: pure molasses I, negative and positive control with PLD1 primers; 7-9: pure molasses I, negative and positive control with PLD2 primers. Figure 1.B: M: marker; 10-12: pure molasses II, negative and positive control with SPS2 primers; 13-15: pure molasses II, negative and positive control with PLD1 primers; 16-18: pure molasses II, negative and positive control with PLD2 primers.

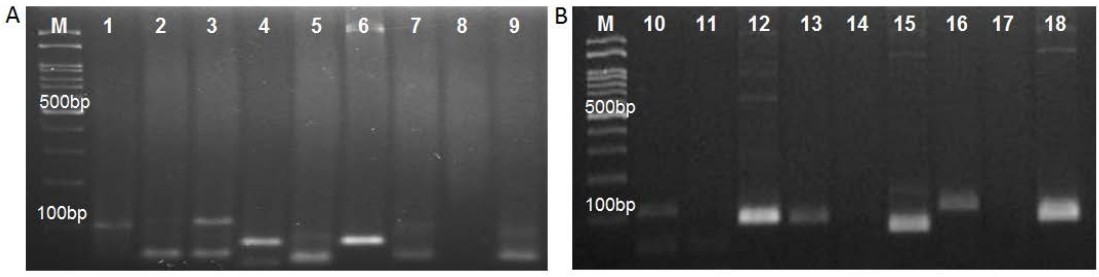


Figure 2. Agarose gel electrophoresis of PCR products for honey mixtures containing rice molasses I using rice primers (SPS2 and PLD1). Figure 2.A: M: marker; 1: pure honey with SPS2 primers; 2-7: honey mixed with 1, 2, 5 10, 20, 50% rice molasses I with SPS2 primers; 8: pure molasses I with SPS2 primers; C-: negative control with SPS2 primers; C+: positive control with SPS2 primers. Figure 2.B: M: marker; 9: pure honey with PLD1 primers; 10-15: honey mixed with 1, 2, 5 10, 20, 50% rice molasses I with PLD1 primers; 16: pure molasses I with PLD1 primers; C-: negative control with PLD1 primers; C+: positive control with PLD1 primers.

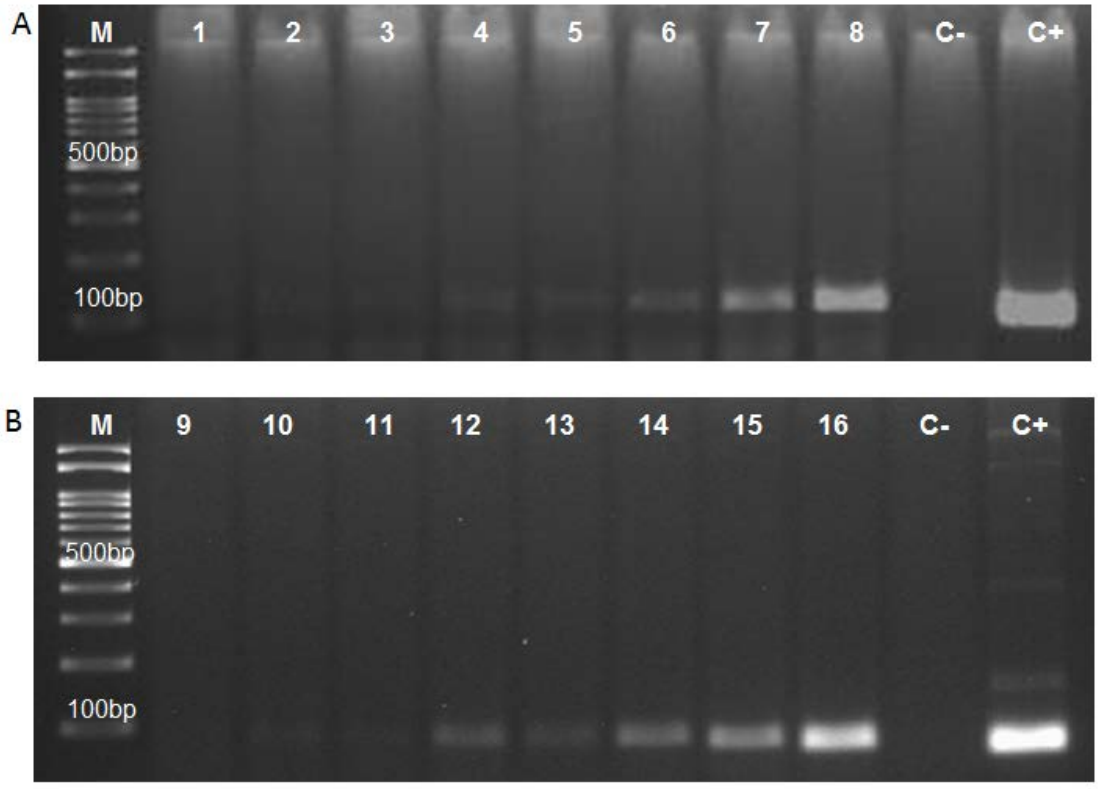


Figure 3. Agarose gel electrophoresis of PCR products for honey mixtures containing rice molasses II using rice primers (SPS2 and PLD1). Figure 3.A: M: marker; 1: pure honey with SPS2 primers; 2-7: honey mixed with 1, 2, 5 10, 20, 50% rice molasses II with SPS2 primers; 8: pure molasses II with SPS2 primers; C-: negative control with SPS2 primers; C+: positive control with SPS2

primers. Figure 3.B: M: marker; 9: pure honey with PLD1 primers; 10-15: honey mixed with 1, 2, 5 10, 20, 50% rice molasses II with PLD1 primers; 16: pure molasses I with PLD1 primers; C-: negative control with PLD1 primers; C+: positive control with PLD1 primers.

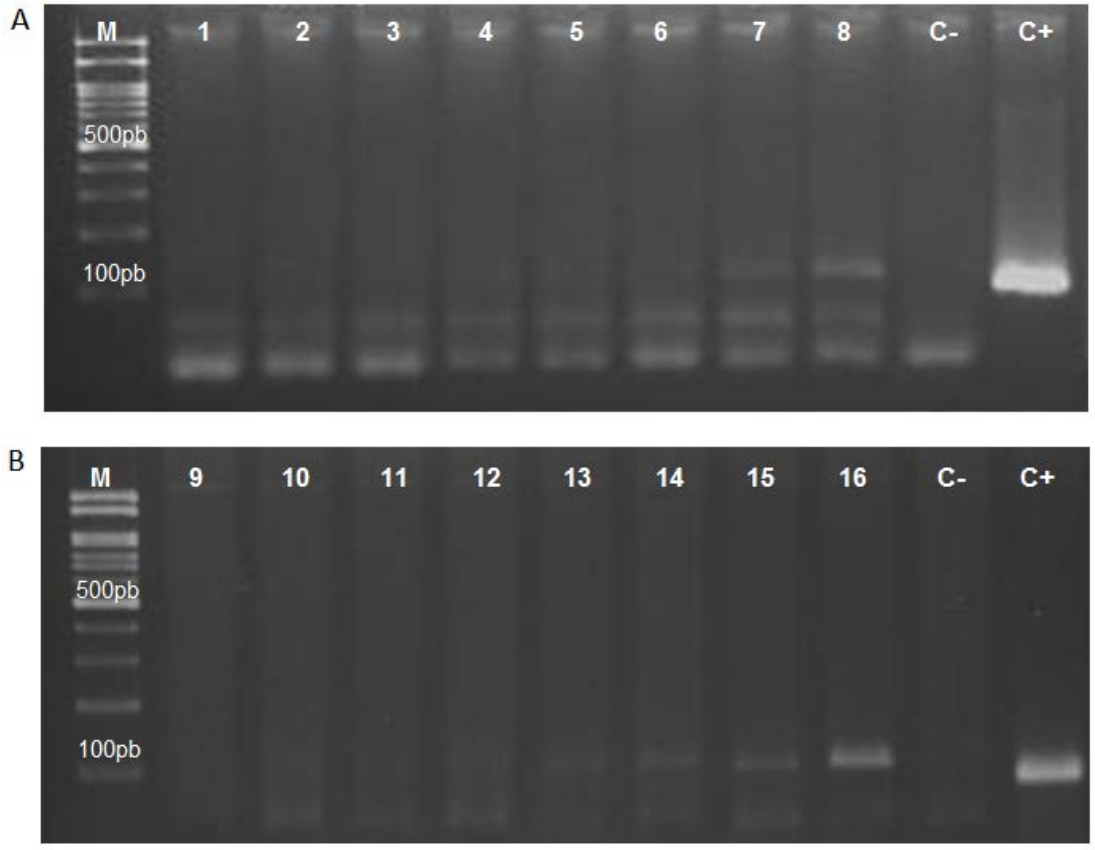
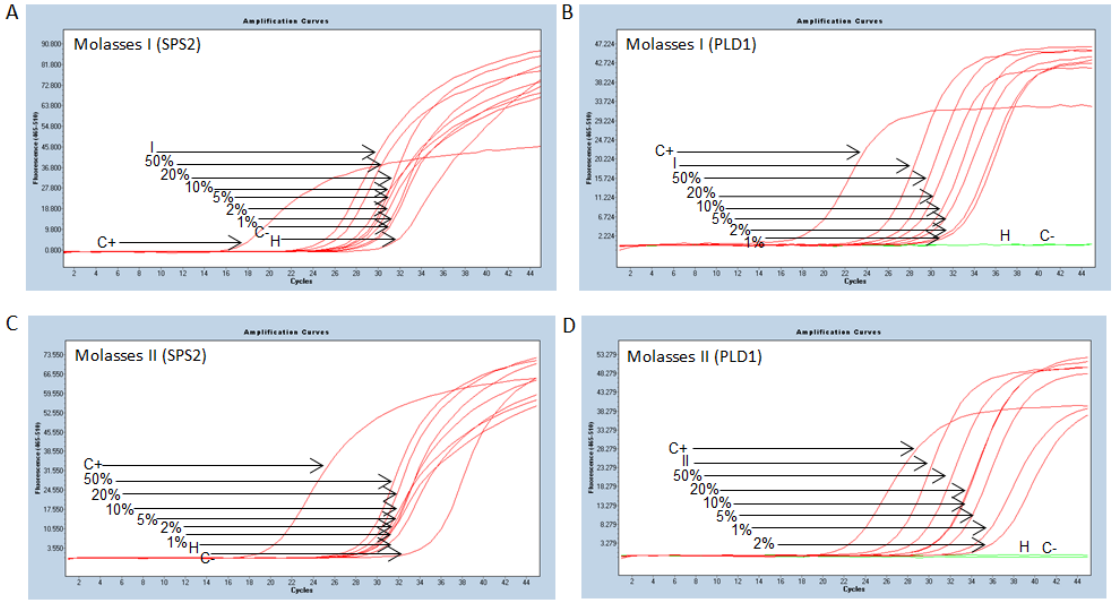


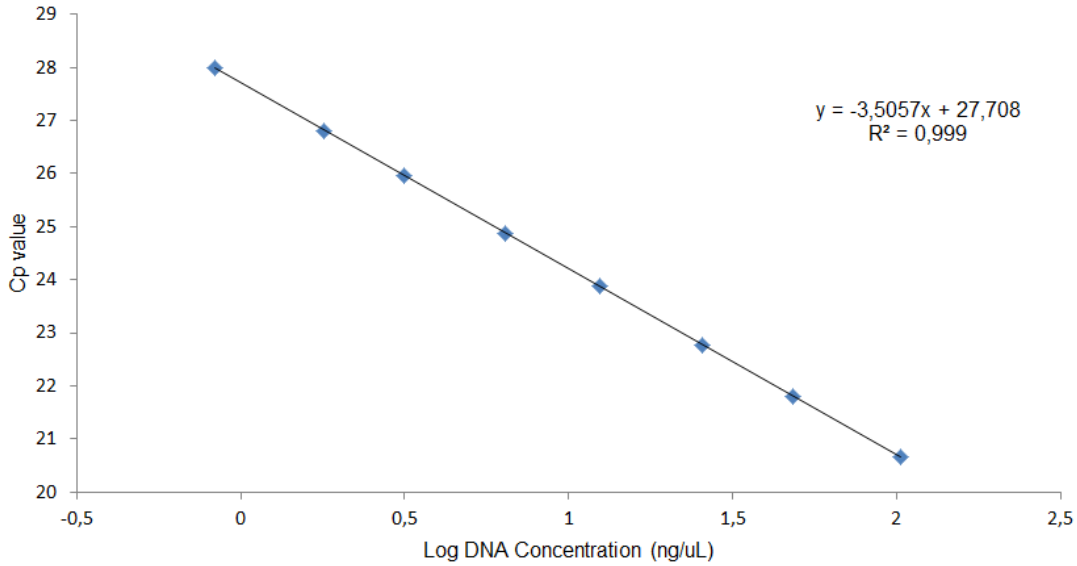
Figure 4. Example of a PCR amplification plot for honey containing different percentages of rice molasses simulating the adulteration (1, 2, 5, 10, 20 and 50%). A: molasses I using SPS2; B: molasses I using PLD1; C: molasses II using SPS2; D: molasses II using PLD1. Abbreviations: I (molasses I), II (molasses II), H (honey), C+ (positive control) and C- (negative control).



504

505 **Figure 5.** Rice genomic DNA standard curve where Cp value was plotted

506 against Log DNA concentration (ng/µL) of DNA standard solution.



507

508 **Highlights**

509 PCR can be applied to detect and quantify the presence of rice molasses in
510 honey

511 PLD1 was the most effective primer for conventional PCR and RT-PCR

512 RT-PCR distinguishes different levels of molasses rice added to honey

513 PCR technique is the best approach in detecting lower levels of adulteration

514

515 **Table 1**. Oligonucleotide primers used in the PCR amplifications

Target	Name	Sequence 5´-3´	Amplicon length (bp)
SPS2	SPS 2-F	GGA TCA TCC CGA AAA GAT CAA C	91
	SPS 2-R	ATG GCA GTG GGA GAG ATT GTG	
PLD1	PLD F(KVM159)	TGG TGA GCG TTT TGC AGT CT	68
	PLD R(KVM160)	CTG ATC CAC TAG CAG GAG GTC C	
PLD2	PLD3959F	GCT TAG GGA ACA GGG AAG TAA AGT T	80
	PLD4038R	CTT AGC ATA GTC TGT GCC ATC CA	

516

517

518 **Table 2**. Cp values (mean values and standard deviation), Log of
519 concentrations and rice DNA concentrations in pure rice molasses I and II, pure
520 orange blossom honey, and mixture of both in different percentages (1, 2, 5, 10,
521 20 and 50%, respectively) simulating the adulteration of honey

Percentage of rice molasses added to pure honey	Cp value	Log of Concentration	Calculated rice DNA concentration (ng/µL)
Rice molasses I			
0% (pure honey)	ND	ND	ND

1%	32.25 (0.32)	-1.773	0.017
2%	32.74 (0.21)	-1.913	0.012
5%	30.68 (0.06)	-1.325	0.047
10%	29.95 (0.17)	-1.117	0.076
20%	28.42 (0.19)	-0.680	0.209
50%	27.45 (0.21)	-0.404	0.395
100% (pure molasses I)	25.56 (0.05)	0.136	1.366
Rice molasses II			
0% (pure honey)	ND	ND	ND
1%	34.65 (1.02)	-2.457	0.003
2%	36.27 (1.65)	-2.919	0.001
5%	32.57 (0.52)	-1.864	0.014
10%	31.27 (0.21)	-1.493	0.032
20%	31.13 (0.04)	-1.453	0.035
50%	29.12 (0.16)	-0.880	0.132
100% (pure molasses II)	26.84 (0.33)	-0.230	0.590
Controls			
Positive Control	20.74 (0.04)	1.760	57.521

Negative Control	ND	ND	ND

522

523

524