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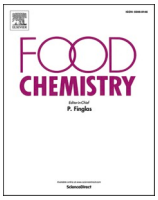

Authentication of incense (*Pittosporum undulatum* Vent.) honey from the Azores (Mel dos Açores) by a novel real-time PCR approach

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ABSTRACT

'Mel dos Açores' is a unique nectar honey produced from the exceptional and diverse flora of the Azores archipelago, categorised as incense honey ('mel de incenso') or multifloral honey ('mel multiflora'). Incense honey should contain over 30 % of pollen grains of *Pittosporum undulatum* Vent. In this work, a real-time PCR method targeting the ITS region was proposed for the first time to detect *P. undulatum* in the honey from the Azores. The approach exhibited high analytical performance, achieving a quantification limit of 0.01 pg of incense DNA. The method was successfully applied to 22 honey samples, from which incense was detected in all 9 monofloral incense honeys and in 5 out of 10 multifloral samples from the Azores. Generally, the quantitative results for incense DNA were in good agreement with the melissopalynological data. Therefore, a simple, cost-effective and reliable tool was herein proposed to authenticate and valorise the Azores honey.

1. Introduction

Honey is a widely consumed natural product owing not only to its taste and nutritional value, but also to its health benefits. Depending on the botanical origin, honey can be classified as monofloral or multifloral, if predominantly produced from a single or multiple plant species, respectively. Usually, honeys from one plant species representing more than 45 % of the total pollen content are classified as monofloral, while multifloral honeys do not evidence one predominant species. However, this classification depends on the pollen source because honeys containing under-represented pollen grains (e.g., lavender, citrus and rosemary) can still be classified as monofloral with pollen proportions of 10–20 %, while honeys having over-represented pollen grains (e.g., chestnut and eucalyptus) can reach pollen frequencies of 70–90 % (Pires, Estevinho, Feás, Cantalapiedra, & Iglesias, 2009; Soares, Amaral, Oliveira, & Mafra, 2017). Honey can also be classified according to its geographical origin, which can be from specific areas within the European Union, holding the labels of Protected Designation of Origin (PDO) and Protected Geographical Identification (PGI) (Machado, Miguel, Vilas-Boas, & Figueiredo, 2020; Soares et al., 2017).

'Mel dos Açores' is a nectar honey produced in the Azores archipelago (Portugal) in accordance with the relevant product specifications, being classified as a PDO honey. 'Mel dos Açores' can be categorised as incense honey ('mel de incenso') or multifloral honey ('mel multiflora'), which are unique products in the world owing to the highly diverse and exceptional flora of the Azores archipelago. The incense honey is made from nectar collected by the western honey bee (*Apis mellifera*) mainly from the flowers of the incense plant *Pittosporum undulatum* Vent., whereas the multifloral honey is made from a mixture of nectars of different flowering plant species. The main defining characteristic of incense honey is its pollen composition, which must contain over 30 % of *P. undulatum* pollen grains, along with smaller amounts of pollen of *Eucalyptus* spp., *Metrosideros excelsa* Gaertner, *Acacia* spp., *Trifolium* spp., *Castanea sativa* Mill., *Rubus* spp., among other species. The multifloral honey of Azores is made from a mixture of nectars of a variety of different flower species (European Commission, 2019). Due to its refined and unique flavour and taste, PDO honey, such as the incense honey from the Azores, is generally perceived as a high-quality product and, consequently, prone to be adulterated through incorrect labelling or admixing with lower-cost and low-quality honeys. Therefore,

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assessing the authenticity of such highly appreciated honeys is a key issue for their valorisation, answering to the increasing consumer demands for transparency (Machado et al., 2020; Soares et al., 2017). Honey authenticity encompasses two main aspects: production and botanical origin. The botanical origin is responsible for the classification of the honey as mono- or multifloral (also PDO and PGI), which is a determinant feature for honey composition and, thus, for its organoleptic properties.

Currently, melissopalynology is the technique used to determine the botanical origin of honey. It relies on the identification and quantification of pollen grains in the pollen sediment of honey. However, this approach is time-consuming, requiring a comprehensive collection of pollen grains and technicians with adequate skills and experience to identify different pollen morphologies, often lacking taxonomic resolution at the genus or species levels (Soares et al., 2017). Physicochemical parameters and chemical markers have been exploited as alternatives to assess the botanical origin of honey (Soares et al., 2017). Particularly, volatile compounds can be considered as a fingerprint for the botanical origin of honey (Kaškonienė & Venskutonis, 2010; Machado et al., 2020; Schievano, Morelato, Facchin, & Mammi, 2013). Recently, benzyl salicylate was suggested as a marker compound of incense honey, also identified in the incense flowers (Machado, Antunes, Miguel, Vilas-Boas, & Figueiredo, 2021). Interestingly, while hotrienol was absent in the incense honey samples, oleic acid was the main component. Other volatile compounds were also identified, namely benzene acetaldehyde, *cis*-linalool oxide (furanoid), *trans*-linalool oxide (furanoid), benzaldehyde, α-eudesmol, α-terpineol and limonene (Machado et al., 2021). Additionally, Machado et al. (2021) found that twelve volatile compounds were enough to fully discriminate eleven honey types (92 %) (bell heather, carob tree, chestnut, eucalyptus, incense, lavender, orange, rape, raspberry, rosemary, sunflower and strawberry tree), according to their botanical origin. However, the chemical composition of honeys can be influenced by environmental conditions and beekeeping practices, often leading to unreliable identification of the botanical origin. In contrast, DNA markers are independent of environmental, physiological, storage and processing conditions, being successfully applied to species identification of several complex and processed botanical matrices (Madesis, Ganopoulos, Sakaridis, Argiriou, & Tsaftaris, 2014; Grazina, Amaral, & Mafra, 2020), including honey (Soares et al., 2017).

DNA-based approaches, such as real-time PCR (Laube et al., 2010; Wu et al., 2017), high resolution melting (HRM) analysis (Soares, Grazina, Costa et al., 2018), droplet digital PCR (You, Mei, Wang, Chen, & Xu, 2021), DNA barcoding (Bruni et al., 2015; Saravanan, Mohanapriya, Laha, Sathishkumar, & Boatwright, 2019) and metabarcoding (Chiara et al., 2021; Utzeri et al., 2018) have demonstrated their feasibility in the identification of pollen from honey. Laube et al. (2010) developed species-specific real-time PCR assays with TaqMan™ probes to detect important plant species in Corsican honey (acacia, broom, citrus, clover, heather, eucalyptus, lavender, linden, oak, olive, rape, rockrose, rosemary, sunflower and sweet chestnut). A similar approach was developed by Wu et al. (2017) to identify the botanical origin of six honey types mainly produced in China (canola, Chinese milkvetch, Chinese chaste tree, locust tree, litchi and longan). Real-time PCR, combined with HRM analysis and DNA barcode markers, was able to differentiate pollen from closely related species of the *Lavandula* genus in honey (Soares, Grazina, Costa et al., 2018). Real-time PCR has also proved to be a powerful approach for identifying the entomological origin of honey, being able to detect the eastern honey bee *Apis cerana* in honey (Soares, Grazina, Mafra et al., 2018). When combined with HRM analysis, it allowed discriminating *A. mellifera* from *A. cerana* (Soares, Grazina, Mafra et al., 2018) as well as differentiating the main *A. mellifera* sub-species found in European honeys (Soares et al., 2019). Moreover, recently, the presence of RNA of plant sources was identified in honey samples, including both plastidial and nuclear transcripts, as well as microRNA sequences, suggesting that they can also be potential markers for honey authentication (Gismondi, Di Marco, & Canini, 2017; Smith et al., 2021).

In the present work, a real-time PCR method targeting the ITS region was proposed for the first time to detect *P. undulatum* species and applied to identify the botanical origin of honey from the Azores. For this purpose, several honey samples, including incense and multifloral honeys from the Azores, were tested using the new molecular method and the results were compared with melissopalynological analysis.

2. Material and methods

2.1. Plant material and sample preparation

Leaves of *P. undulatum* were collected in the Azores archipelago (Fig. 1). Other plant species (including some endemic), usually mentioned in Azorean honey, were also collected in the Azores and mainland Portugal for cross-reactivity tests, including *Eucalyptus* spp., *Acacia* spp., *Trifolium* spp., *Castanea sativa* Mill., *Hydrangea macrophylla*, *Rhododendro indicum*, *Hedychium gardnerianum*, *Pericallis malvifolia*, among others (Table 1). All collected leaves were dried and posteriorly ground using a knife mill Grindomix GM200 (Retsch, Haan, Germany) with decontaminated knives and blender containers. The ground leaves were stored at room temperature (under desiccation) until DNA extraction.

Honey samples from four Azorean islands (São Miguel, Faial, Terceira and Pico) were acquired in local Azorean markets, mainland Portuguese markets and directly from honey producers (Table 2). The samples included nine incense honeys (H1-H9), ten multifloral honeys (*H*10-H19), of which one had clover (H19) predominance (Fig. 1). Additionally, a sample of clover honey from the Azores (H20) and two samples of monofloral honeys of rosemary (H21) and chestnut (H22) from typical Portuguese regions were acquired. All the honey samples from the Azores were subjected to melissopalynological analysis performed by an outsourced Portuguese laboratory (Apismaia, Portugal) (Table S1 Supplementary material).

All honey samples were submitted to a pre-treatment to remove possible interfering compounds from the matrix before DNA extraction, as described by Soares, Grazina, Costa et al. (2018), Soares, Grazina, Mafra et al. (2018) with minor modifications. Briefly, 50 g (four tubes with 12.5 g each) of each honey sample were frozen (−80 °C) overnight. After adding distilled water to each tube up to a volume of 45 mL, the mixture was vigorously vortexed and heated at 45 °C for 5 min. The tubes were centrifuged (5500×*g* for 20 min, 4 °C) and the supernatant was discarded. The pellet was suspended in 1 mL of distilled water and transferred to 2 mL reaction tubes. The four reaction tubes were frozen (−20 °C) overnight, heated at 45 °C for 5 min and centrifuged (17,000×*g* for 10 min, 4 °C). Again, the pellets were resuspended in distilled water and combined in a single reaction tube (2 mL) that was

Fig. 1. Incense (*P. undulatum*) flowers and honey samples used in this work.

Table 1
Results of PCR amplification targeting the ITS region of *P. undulatum* and a universal eukaryotic DNA region (18S rRNA gene) of several relevant plant species for cross-reactivity testing.

Species	Common name	Origin	PCR (18S rRNA)	PCR (ITS)
Pittosporum undulatum	Incense	S. Miguel	+	+
P. undulatum	Incense	S. Miguel	+	+
P. undulatum	Incense	S. Miguel	+	+
Eucalyptus spp.	Eucalyptus	S. Miguel	+	−
Acacia spp.	Acacia	S. Miguel	+	−
Trifolium spp.	Clover	Barcelos	+	−
Castanea sativa	Chestnut	Barcelos	+	−
Hydrangea macrophylla	Hydrangea	Porto	+	−
Rhododendro indicum	Azalea	Barcelos	+	−
Pericallis malvifolia	Azorean Pericallis	S. Miguel	+	−
Metrosideros excelsa	Metrosidero	Porto	+	−
Banksia integrifolia	Coast banksia	S. Miguel	+	−
Rubus spp.	Brambles	S. Miguel	+	−
Musa spp.	Banana tree	S. Miguel	+	−
Psidium gujava	Common guave	S. Miguel	+	−
Hedychium gardenrianum	Ginger lily	S. Miguel	+	−
Persea americana	Avocado tree	S. Miguel	+	−
Salvia rosmarinus	Rosemary	S. Miguel	+	−
Physalis peruviana	Physalis	S. Miguel	+	−
Passiflora edulis	Passion fruit tree	Barcelos	+	−
Actinidia deliciosa	Kiwi tree	Marco de Canaveses	+	−
Myrica faya	Firetree	Porto	+	−
Camellia spp.	Camellia	S. Miguel	+	−
Vicia faba	Faba Camellia bean	Marco de Canaveses	+	−
Zantedeschia aethiopica	Calla Lily	Porto	+	−
Psidium cattleyanum	Cattley guava	Leiria	+	−

centrifuged (17,000×*g* for 10 min, 4 °C). The supernatant was discarded, and the pellet was stored at −20 °C until DNA extraction.

2.2. DNA extraction

DNA was extracted from the plant specimens and the honey samples with the NucleoSpin® Plant II kit (Macherey-Nagel, Düren, Germany) using 100 mg of leaf material or the frozen honey pellet, according to the manufacturer's instructions with minor modifications, as described by Soares, Amaral, Oliveira, and Mafra (2015). The DNA extracts were immediately stored at −20 °C until further analysis.

The yield and purity of DNA extracts were assessed by UV spectrophotometry using a Take3 micro-volume plate accessory on a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc., Vermont, USA). The nucleic acid protocol, with sample type defined for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Vermont, USA), was applied to the measured absorbency data at 260, 280 and 320 nm.

The quality of the DNA extracts was further assessed by electrophoresis with 1 % agarose gel stained with 1 × Gel Red (Biotium, CA, USA) and performed in 1 × SGTB buffer (GRISP, Porto, Portugal) for 20–25 min at 200 V. The agarose gel was visualised with a UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA) and a digital image was obtained with Image Lab software version 5.1 (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Target gene selection, oligonucleotide primers and probes

A complete DNA sequence of the internal transcribed spacer 1 (ITS1), partial sequence and 5.8S ribosomal RNA gene and internal transcribed spacer 2 (ITS2) from *P. undulatum* was used for primer and probe design. Two primer sets, namely ITS_Pu-F2/ITS_Pu-R2 for qualitative and real-time PCR and ITS_Pu-FS/ITS_Pu-RS for sequencing, were designed using the software Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), as well as one hydrolysis probe, labelled with fluorescein (FAM) as the fluorescent reporter and black hole quencher BHQ-1 as the quencher (ITS_Pu-P) (Table 3). The absence of hairpins and self-hybridization was assessed by the OligoCalc software (http://www.

Table 2
Honey samples analysed by qualitative PCR and real-time PCR targeting the ITS region of incense and respective incense pollen content as determined by melissopalynology.

Code	Honey sample (labelled information)	Origin	PCR[a]		Real-time PCR		Incense pollen (%)
			18S rRNA	ITS	Cq[b] (mean ± SD)	DNA (pg) (mean ± SD)	
H1	Incense	S. Miguel	+	+	28.76 ± 0.40	1.81 ± 0.54	67
H2	Incense	Faial	+	+	29.42 ± 0.17	1.13 ± 0.13	50
H3	Incense	Faial	+	+	27.81 ± 0.21	3.38 ± 0.50	49
H4	Incense PDO	S. Miguel	+	+	28.06 ± 0.16	2.85 ± 0.32	73
H5	Incense PDO	S. Miguel	+	+	28.05 ± 0.53	1.09 ± 0.21	52
H6	Incense	Pico	+	+	27.42 ± 0.35	1.63 ± 0.28	55
H7	Incense	S. Miguel	+	+	31.35 ± 0.80	0.339 ± 0.170	62
H8	Incense PDO	Terceira	+	+	32.14 ± 0.20	0.180 ± 0.025	45
H9	Incense	Terceira	+	+	27.03 ± 0.40	5.85 ± 1.52	62
*H*10	Multifloral	Faial	+	−	< LOD[c]		ND[d]
*H*11	Multifloral	S. Miguel	+	+/-	34.57 ± 0.76	0.038 ± 0.015	25
H12	Multifloral	Pico	+	+/-	< LOD		7
H13	Multifloral	S. Miguel	+	−	< LOD		4
H14	Multifloral PDO	Terceira	+	+	35.74 ± 0.39	0.016 ± 0.005	3
H15	Multifloral	S. Miguel	+	+	< LOD		6
H16	Multifloral PDO	S. Miguel	+	+	< LOD		8
H17	Multifloral PDO	S. Miguel	+	+	36.03 ± 0.66	0.016 ± 0.007	7
H18	Multifloral	S. Miguel	+	+	31.01 ± 0.09	0.414 ± 0.025	19
H19	Multifloral[e]	S. Miguel	+	+	28.56 ± 0.16	2.08 ± 0.21	46
H20	Clover	S. Miguel	+	−	< LOD		<3
H21	Chestnut	Sabroso de Aguiar	+	−	< LOD		NA[f]
H22	Lavender	Trás-os-Montes	+	−	< LOD		NA

[a] (+), detected PCR products; (−), not detected; (+/−), very faint PCR products; [b]Cq, quantitative cycle (mean value ± standard deviation); [c]LOD, limit of detection; [d]ND, not detected; [e]Multifloral with predominance of clover; [f]NA, not analysed.

basic.northwestern.edu./biotools/oligocalc.html). The specificity of the oligonucleotide primers was assessed using the Primer-BLAST tool that enabled revealing potential homologies between all sequences available in the GenBank database (Fig. S1, Supplementary material).

To assess the amplification capacity of the DNA extracts, universal eukaryotic primers targeting the conserved nuclear 18S rRNA gene (Table 3) were used. All primers and probe were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

2.4. Qualitative PCR

PCR amplification was carried out in 25 μL of total reaction volume containing 20 ng to 0.002 pg of DNA, buffer (67 mM Tris-HCl, pH 8.8, 16 mM $(NH_4)_2SO_4$, 0,01 % Tween 20), 3 mM of $MgCl_2$, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience GmbH, Germany), 200 nM of each primer (Table 3) and 200 μM of dNTP (Grisp, Porto, Portugal). The reactions were carried out in a MJ Mini™ Gradient Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA), with the following temperature programs: initial denaturation at 95 °C for 5 min; 33 or 40 cycles (for 18SRG-F/18SRG-R or ITS_Pu-F2/ITS_Pu-R2 primers, respectively) of amplification at 95 °C for 30 s, 65 °C or 64 °C (for 18SRG-F/18SRG-R or ITS_Pu-F2/ITS_Pu-R2 primers, respectively) for 30 s and extension at 72 °C for 30 s; and a final extension at 72 °C for 5 min.

PCR products were verified by electrophoresis in a 2 % agarose gel stained with 1 × GelRed and carried out in 1 × SGTB (Grisp, Porto, Portugal), for 25–30 min at 200 V. The agarose gel image was collected under a UV light tray GelDoc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA) and recorded with Image Lab software version 5.2.1 (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Sequencing

PCR fragments amplified with the primer pair ITS_Pu-FS/ITS_Pu-RS (Table 3) were purified using the GRS PCR & Gel Band Purification Kit (GRISP, Porto, Portugal) to remove any possible interfering components. Afterwards, the purified products were sent to a specialised facility (Eurofins Genomics, Ebersberg, Germany) for Sanger sequencing. Each target fragment was sequenced twice, performing the direct sequencing of both strands in opposite directions to allow the production of two complementary sequences of high quality. Sequencing data were aligned using the software BioEdit v7.2.5 (Ibis Biosciences, Carlsbad, CA, USA) and the electropherograms were analysed with FinchTV (Geospiza, Seattle, WA, USA).

2.6. Real-time PCR

The real-time PCR amplifications were carried out in 20 μL of total reaction volume, containing 2 μL of DNA (10 ng-0.01 pg), 1 × SsoFast Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 300 nM of each primer (ITS_Pu-F2/ITS_Pu-R2) and 150 nM of the probe (ITS_Pu-P) (Table 3). A fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) was used with the following conditions: 95 °C for 5 min, 50 cycles at 95 °C for 10 s and 65 °C for 40 s, with the collection of fluorescence signal at the end of each cycle. The data evaluation, from each real-time PCR assay, was done using the software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR assays were performed in at least two independent runs using $n = 4$ replicates in each one.

A calibration curve was constructed using 10-fold serially diluted incense DNA extracts (10 ng-0.01 pg), which allowed determining the absolute limits of detection (LOD) and quantification (LOQ). The acceptance criteria established for real-time PCR assays were the PCR efficiency between 90 and 110 %, the slope within −3.6 and −3.1 and the correlation coefficient (R^2) above 0.98 (Bustin et al., 2009; ENGL, 2015). The LOD was considered as the lowest amplified level for 95 % of the replicates, while the LOQ was established as the lowest amplified level within the linear dynamic range of the calibration curve, which should cover a minimum of 4 orders of magnitude and should extend to ideally 5 or 6 $\log_{10}$ concentrations (Bustin et al., 2009; ENGL, 2015).

3. Results and discussion

In the present work, a new DNA marker was identified in the ITS region of *P. undulatum*, which allowed the development of a species-specific PCR assay as a screening tool to detect incense and a quantitative real-time PCR method with a specific hydrolysis probe to confirm and estimate incense DNA content. Both approaches were then applied to commercial honey samples from Azores with known pollen content, determined by microscopic analysis, and the results were compared and further validated.

3.1. Evaluation of extracted DNA

Considering the complexity of the honey matrix, mainly composed of different sugars and other substances, such as organic acids, polyphenols, pigments, enzymes and solid particles as waxes, in which the pollen grains are minor components, it is crucial to perform a sample pre-treatment to isolate/concentrate pollen as much as possible free from matrix interferents. Therefore, prior to DNA extraction, honey samples were subjected to several washing, freezing and centrifuging steps to remove most interfering compounds from the matrix, as described by Soares et al. (2018) with some minor modifications. The selection of the DNA extraction methods was also a critical step, which considered our previous experience with other honey samples (Soares et al., 2015; 2018), as well as with herbal medicines (Grazina, Batista, Amaral, Costa, & Mafra, 2022), for which the Nucleospin Plant II kit (Macherey-Nagel, Düren, Germany) provided suitable extracts for real-time PCR amplification.

The DNA extracts from plant material (leaves), including incense (*P. undulatum*) used for method development, revealed appropriate concentrations (6.3–197.8 ng/μL) and purities ($A_{260}/A_{280} = 1.5–2.2$) for further PCR amplification. Similarly, the DNA extracts from honey samples achieved adequate yields (17.4–228.7 ng/μL) and purities ($A_{260}/A_{280} = 1.4–2.2$). These values are in good agreement with other studies reporting on DNA extraction of honey samples using identical (Soares et al., 2018) and different (Bruni et al., 2015) methods.

The amplification capacity of all DNA extracts was assessed by PCR using universal primers targeting the 18S rRNA gene (Table 3), which was successful for all plant and honey extracts, eliminating eventual

Table 3
Primers and probe used in qualitative and real-time PCR.

Target region	Primer/ probe	Sequence (5′-3′)	Amplicon (bp)	GenBank
ITS	ITS_Pu-FS	AAGTTGCGCCCGAAGCCATTA	244	HM116994.1
	ITS_Pu-RS	ATCGCTCTGGCGGCATTCGA		
	ITS_Pu-F2	AACCCTCCCTATCCCTGATC	99	HM116994.1
	ITS_Pu-R2	GAGGACTCGCATTTGGGACAA		
	ITS_Pu-P	FAM-CAATATCCGCCCCCCGCACTCGGTA-BHQ1		
18S rRNA gene	18SRG-F	CTGCCCTATCAACTTTCGATGGTA	113	Costa, Oliveira, and Mafra (2013)
	18SRG-R	TTGGATGTGGTAGCCGTTTCTCA		

false-negative results (Tables 1 and 2).

3.2. Species-specific PCR

In silico analysis was performed for primer design targeting the *trnL* (NCBI accession No. MF503614.1) and the internal transcriber spacer (ITS) regions of *P. undulatum* (NCBI accession No. HM116994.1). Two sets of primers were designed for each target region, from which only the pair Pu_ITS-F2/Pu_ITS-R2 (Table 3), producing 99-bp amplicons, revealed full specificity for *P. undulatum*, after testing several plant species, mostly endemic to the Azores archipelago (Table 1). The PCR results using serially diluted DNA achieved a sensitivity down to 2 pg of incense (Fig. S2, Supplementary material).

The sequencing results using the primer pair ITS_Pu-FS/ITS_Pu-RS, designed to obtain a 244-bp fragment that encompasses the target 99-bp amplicon, revealed almost full homology with the sequence retrieved from NCBI (Fig. S3, Supplementary material), confirming the species-specific PCR results.

3.3. Real-time PCR

Thereafter, a real-time PCR assay using a specific hydrolysis probe was developed targeting the ITS region of *P. undulatum*. Fig. 2A and 2B show the amplification curves and respective calibration curve of serially diluted incense DNA of one example assay. The assays exhibited a slope of −3.397 ± 0.160, a PCR efficiency of 97.4 ± 6.4 %, a coefficient

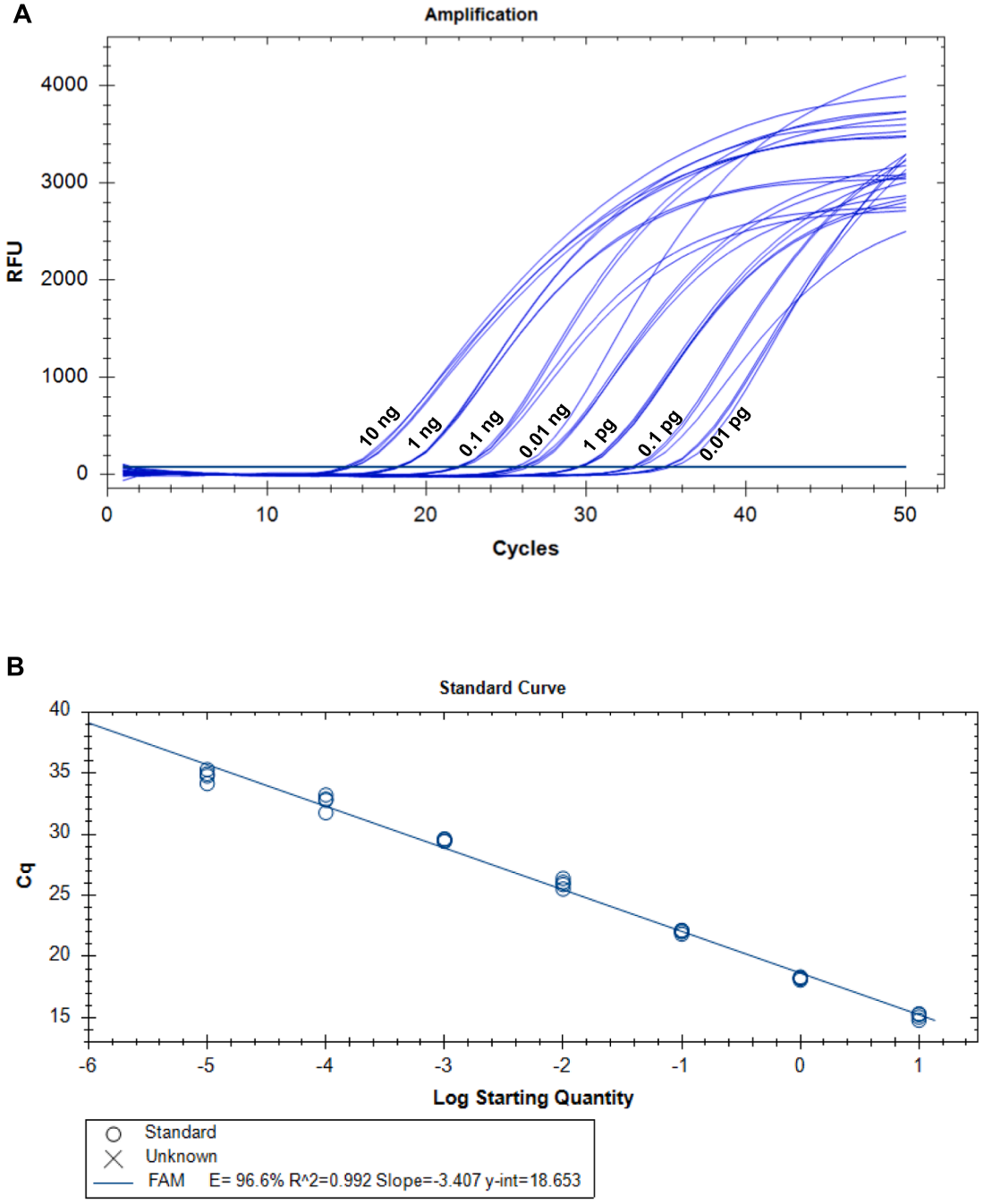

Fig. 2. Amplification (A) and calibration (B) curves of a real-time PCR assay, targeting the ITS region of 10-fold serially diluted incense (*Pittosporum undulatum*) DNA (10 ng-0.01 pg) (n = 4 replicates).

of correlation (R^2) of 0.991 ± 0.003 and a dynamic range of 6 magnitude orders (10 ng-0.01 pg), indicating that all parameters were within the acceptance criteria established for real-time PCR assays (Bustin et al., 2009; ENGL, 2015). The reached LOD was 0.01 pg (0.005 pg/μL) of incense DNA since it was the lowest level amplifying all the replicates, being also established as the LOQ, as it was within the linear dynamic range of the calibration curve (Fig. 2B) (ENGL, 2015). The sensitivity of the real-time PCR assay was substantially higher (200-fold) than that obtained by end-point PCR and by other authors (100-fold) that achieved LOD values within 0.5–5 pg/μL for six plant species by real-time PCR targeting chloroplastidial genes (Wu et al. (2017). Similar sensitivity levels can also be found in the literature using real-time PCR with TaqMan probes targeting the ITS region for detecting botanicals in herbal products (0.01 pg/μL) (Grazina, Amaral, Costa, & Mafra, 2020; Grazina, Amaral, Costa, & Mafra, 2021) or even plant species used as food ingredients, such as sesame and flaxseed (1 mg/kg) (López-Calleja, Cruz, Martín, González, & García, 2015a), and cashew and macadamia (0.1 mg/kg) (López-Calleja, Cruz, Martín, González, & García, 2015b). Therefore, nuclear ITS markers, besides being very specific, are also able to provide highly sensitive detection methods, comparable to chloroplastidial markers. In addition to the high sensitivity and specificity, the real-time PCR approach proposed herein is also easier and faster to implement than other DNA-based methods, such as DNA-barcoding relying on sequencing (Bruni et al., 2015; Saravanan et al., 2019).

3.4. Application to honey samples and assay validation

After performing a market search in the Azores, in collaboration with local official entities and producers, 20 samples were identified as labelled "Mel dos Açores", including 9 monofloral from incense and 10 multifloral. As expected from labelled information, all the incense honey samples (H1-H9) were positive for incense detection by qualitative PCR (Fig. S4, Supplementary information) and this result was confirmed by real-time PCR (Table 2). Regarding the multifloral honeys (H10-H19), 5 out of 10 were confirmed positive for the incense detection, which is consistent with the widespread distribution of incense in the Azores archipelago (https://acores.flora-on.pt/#/1incenso). As also expected, the 2 honeys sampled in mainland Portugal (H21 and H22) were negative to incense. Overall, the quantitative results of real-time PCR were in good agreement with the melissopalynological data as the highest incense DNA values (0.180–5.85 pg) were mostly found in the monofloral honey samples containing the highest proportions of incense pollen grains (45–73 %). Likewise, multifloral honey samples, mostly with much lower amounts of incense DNA, contained minor or trace levels of incense pollen grains. Surprisingly, the multifloral honey sample H19, which was labelled as having predominance of clover, contained relatively high amounts of both incense DNA (2.08 ± 0.21 pg) and pollen grains (46 %) (Table 2), which suggests that it should have been classified as a monofloral incense honey (>30 % of incense pollen) instead of multifloral (European Commission, 2019). Fig. 3 provides an example image the melyissopalynological analysis of a honey sample, where pollen of *Pittosporum undulatum* can be identified among pollens of other species. Despite the overall good agreement, it is not possible to draw any correlation between the amounts of incense DNA and pollen, thus hampering to discriminate monofloral incense from multifloral honey. This would require suitable calibrants, probably using reference mixtures of honey spiked with known amounts of incense pollen, which should be an issue of further research. Samples H10 and H20 labelled as multifloral and clover, respectively, were both classified as clover based on the melissopalynological analysis (Table S1, Supplementary material). All other Azores' honey samples are according to their labelled classification.

4. Conclusions

In the present work, a real-time PCR approach using a TaqMan probe to target the ITS region of *P. undulatum* was developed to specifically detect and quantify incense DNA in honey. The herein identified ITS marker for authenticating incense honey showed high specificity against several plant species, including endemic species from the Azores archipelago, and high sensitivity, down to 0.01 pg of DNA, for *P. undulatum*. The real-time PCR approach exhibited high analytical performance as revealed by the parameters of slope, PCR efficiency and R^2, which were all within the acceptance criteria, with a dynamic range covering 6 orders of magnitude (10 ng – 0.01 pg) and a LOQ of 0.01 pg. The method was successfully applied to 22 honey samples, from which incense was detected in all 9 monofloral incense honeys and in 50 % of the multifloral samples from the Azores. Generally, the quantitative results for

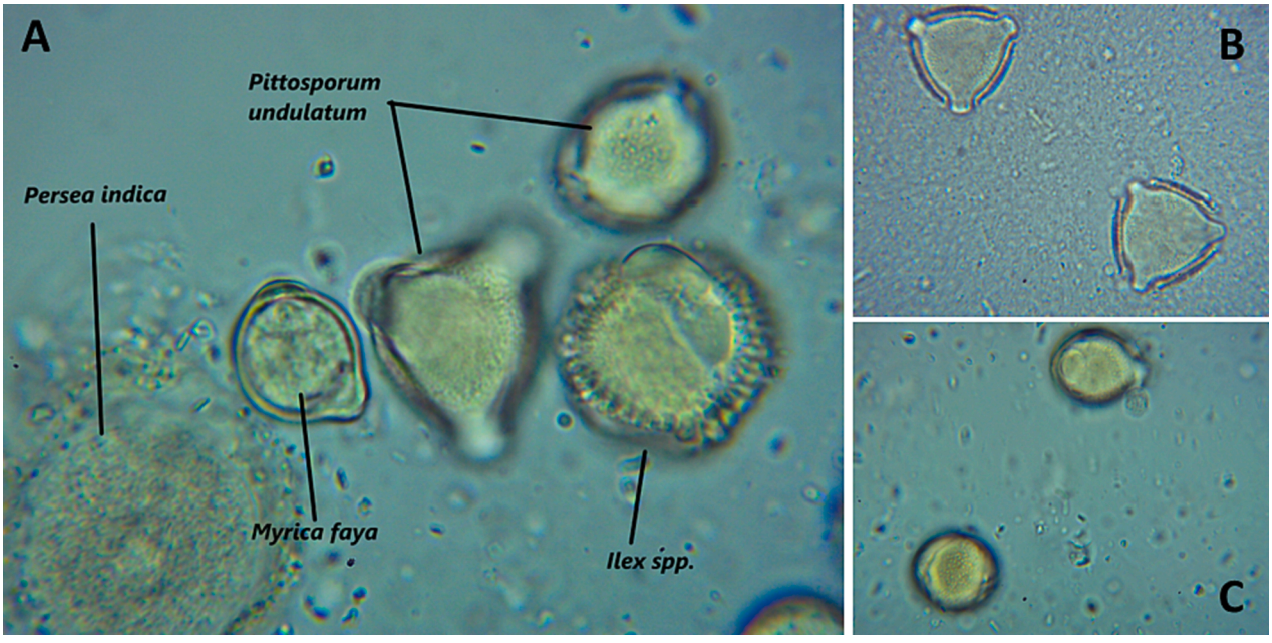

Fig. 3. Pollen grains of an example sample under microscope (×1000). *Pittosporum undulatum* pollen among other pollen species (A); polar view of *P. undulatum* pollen (B); equatorial view of *P. undulatum* pollen (C).

incense DNA were in good agreement with the melyssopalynological data, showing that all samples complied with their labelled statements, except for 2 multifloral honey samples that should have been classified as monofloral of clover and monofloral of incense.

To our knowledge, this is the first DNA-targeted approach to detect incense pollen in honey, which can be a powerful tool for simple, cost-effective and reliable botanical authentication of honey from the Azores archipelago.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.135492.

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