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Detection of sugar syrup adulteration in UK honey using DNA barcoding

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ABSTRACT

Honey is a valuable and nutritious food product, but it is at risk to fraudulent practices such as the addition of cheaper syrups including corn, rice, and sugar beet syrup. Honey authentication is of the utmost importance, but current methods are faced with challenges due to the large variations in natural honey composition (influenced by climate, seasons and bee foraging), or the incapability to detect certain types of plant syrups to confirm the adulterant used. Molecular methods such as DNA barcoding have shown great promise in identifying plant DNA sources in honey and could be applied to detect plant-based sugars used as adulterants. In this work DNA barcoding was successfully used to detect corn and rice syrup adulteration in spiked UK honey with novel DNA markers. Different levels of adulteration were simulated (1 – 30%) with a range of different syrup and honey types, where adulterated honey was clearly separated from natural honey even at 1% adulteration level. Moreover, the test was successful for multiple syrup types and effective on honeys with different compositions. These results demonstrated that DNA barcoding could be used as a sensitive and robust method to detect common sugar adulterants and confirm syrup species origin in honey, which can be applied alongside current screening methods to improve existing honey authentication tests.

1. Introduction

Honey is a naturally sweet substance produced by honeybees, used widely as a food product or natural sweetener. Honey characteristics vary greatly depending on the botanical origins of nectar sources; which are linked to the geographic location of the apiaries and the season of harvest, resulting in honeys with different flavours, colours and composition (Lazarević et al., 2017). Globally, the consumption of honey is steadily increasing, driven not only by the growth of world population but by the increased preference towards natural and healthier food products, causing a significant increase in international honey trade (García, 2018). Consequently, in the UK over 50,000 tonnes of honey was imported in 2023 to supplement the smaller local production and supply the growing consumer demand (HMRC, 2023). At the same time, similarly to other high valued food products, honey is vulnerable to food fraud and concerns have been raised over the product authenticity, generating investigations in the EU in 2016 which revealed that 14.2% of honey ($n = 893$) sampled at different points of the supply chain (majority retail samples) and tested for C4 sugar adulteration were suspicious of non-compliance to the criteria of the EU Honey directive 2001/110/EC (Aries, et al., 2016; EU, 2002). Strikingly, the most recent EU report published in 2023 found that 46% of honey samples imported into the EU ($n = 320$) and tested for multiple markers of sugar adulteration were suspicious of non-compliance, demonstrating that honey adulteration is becoming an issue of increasing concern (Ždiniaková et al., 2023). It is important to note that the observed increase in suspicious non-compliances is based on arrays of highly interpretive tests in addition to the traditional C4 sugar detection method, therefore further investigation is needed to assess the robustness of the findings.

Although honey consists mostly of various sugar forms (fructose, glucose, maltose, and sucrose), it also contains other minor components, including essential vitamins and minerals, amino acids, proteins, and phenolic compounds, which positively impacts the nutritional and economical value of honey (da Silva et al., 2016). Related sugar syrups are derived from plants and obtained by processing juice or modifying the starch with heat, enzyme or acid treatments (Fakhlaei et al., 2020; Guler et al., 2014). They generally have a lower economic and nutritional value than honey but possess a similar appearance and sweet

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taste, therefore could be used to adulterate pure honey for economic gain. Thus, impacting the flavour, chemical composition and nutritional properties of the honey and resulting in an inferior product (Soares et al., 2017). Typically syrups derived from corn, sugar cane, sugar beet, rice and wheat could be used for direct sugar adulteration of honey, which depends on the market availability (Trifković et al., 2017). Sugar adulteration of honey can also occur indirectly from the unethical feeding of bees using sugar syrups during a nectar flow in order to increase the yield (Stefas et al., 2022).

Traditional methodology for sugar adulteration detection in honey relies on assessing the sugar composition using methods such as high-performance liquid chromatography (HPLC). The Honey legislation for England (Honey (England) Regulations, 2015) and the Codex Standard 12–1981 for honey (Codex Alimentarius, 1981) legislate the minimum quantity of fructose and glucose (no less than 60 g/100g) and maximum levels of sucrose (no more than 5 g/100g) in honey, with deviations allowed for certain honey types such as honeydew, lavender, borage and *Citrus* spp. amongst others due to the natural differences in sugar composition in these nectar sources. However, as some sugar syrups contain a similar sugar composition to the nectar that bees forage on, more advanced techniques are needed to detect sugar adulteration in honey. Most of the modern present methodology for exogenous sugar detection in honey are based on stable carbon isotope ratio analysis (SCIRA), which relies on identifying the presence of adulterant C4 plants by assessing the $^{13}C/^{12}C$ ratio (White, 1992). This is based on the fact that honey should be almost entirely derived from plants which have the C3 (Calvin cycle) photosynthetic pathway, whereas plants like corn and sugar cane possess the C4 (Hatch-Slack) system (Tosun, 2013). C4 plants have a higher ^{13}C value which becomes apparent when honey is adulterated with an abundance of corn or cane syrup (Tosun, 2013). Nevertheless, for syrups derived from C3 plants, such as rice and sugar beet, SCIRA is not suitable, thus alternative methods are required. It was indicated in the 2023 EU technical report that SCIRA methods (AOAC method 991.41) were not effective in detecting honeys suspicious of non-compliance, suggesting that sugar syrups from corn or sugar cane are no longer being used to adulterate honey imported for European consumption (Ždiniaková et al., 2023).

Alternative methods for sugar adulteration detection include assessing the chemical composition of honey with the presence of specific markers such as mannose, difructose anhydride (DFA) and 2-Acetylfuran-3-glucopyranoside (AFGP) (Misslera et al., 2016; Montilla et al., 2006; Xue et al., 2013), applying sugar characterisation (Cordella et al., 2005; Morales et al., 2008) or honey profiling using nuclear magnetic resonance (NMR) (Rhee et al., 2023; Spiteri et al., 2015). However, these methods are likely to be affected by the large variation in the physical and chemical compositions of natural honey types with diverse origin, causing unreliable or misinterpreted results (Soares et al., 2017). Extensive databases need to be validated to accurately represent the fingerprint of different honey types and common blends, which is a difficult task to achieve.

DNA based methods have been widely used in food authentication to identify the presence of plant species in various food products or raw ingredients (Madesis et al., 2014). In honey there are multiple sources of nucleic acids, including plant DNA from the floral forage, microbial DNA, and animal DNA derived from the honeybees (Wirta et al., 2021). Although DNA based methodology has predominantly focused on the botanical authentication of honey (Laube et al., 2010; Lopes et al., 2023; McDonald et al., 2018; Soares et al., 2018; Wu et al., 2017), it has also been shown that residual plant DNA from sugar syrup addition can be successfully identified in particular honey samples when they were mixed with rice or corn molasses (Sobrino-Gregorio et al., 2019; Truong et al., 2022). Nevertheless, this technique has not yet been tested on a wider range of syrups and honey types or applied for other common sugar syrup adulterants. It is vital to assess the reliability of the method in possible real-life situations, where different adulterant syrups can be used on any type of honey.

This study aimed to develop a robust DNA barcoding method for exogenous sugar adulteration detection in honey, focusing on rice, corn, and sugar beet syrup detection. The method was tested on multiple syrups and honey types to assess the specificity and sensitivity of fraud detection. Sugar composition analysis using HPLC was carried out alongside the DNA tests, to complement the method with supplementary data.

2. Materials and methods

2.1. Sample collection and preparation

2.1.1. Honey samples

17 honey samples (H01 - H17) were collected from bee farmers around the UK, representative of different seasons and floral sources (Shehata et al., 2024). Additionally, 4 honey samples of UK origin were purchased commercially (CH1 – CH4) from supermarkets and online retailers. Table 1 includes the types of honey, and the type of flora predominantly available to bees in the area surrounding the hives. When a single flora type labels the type of honey, this is not necessary an indication the honey is monofloral and it is information obtained from the honey producers. The samples were stored at room temperature, away from light.

Table 1
Honey sample information.

Sample #	Honey description	Bee forage [a]	Season	Area
H01	woodland	Woodland trees and nearby flowers, including lime (*Tilia*), horse chestnut (*Aesculus hippocastanum)* and sweet chestnut (*Castanea sativa*).	Summer	Yorkshire
H02	sycamore	Predominantly *Acer pseudoplatanus* with a bit of hawthorn (*Crataegus monogyna)* and bean (Fabaceae)	Spring	Yorkshire
H03	phacelia	*Phacelia tanacetifolia*	Spring	Yorkshire
H04	ivy	*Hedera helix*	Autumn	Yorkshire
H05	Himalayan balsam	*Impatiens glandulifera*	Autumn	Yorkshire
H06	spring set	Mixture of farmed and wild spring flowers	Spring	Yorkshire
H07	borage	*Borago officinalis*	Summer	Warwickshire
H08	buckwheat	*Fagopyrum esculentum*	Autumn	Yorkshire
H09	meadowfoam	*Limnanthes alba*	Summer	Warwickshire
H10	sea lavender	*Limonium vulgare*	Summer	Norfolk
H11	heather	*Calluna vulgaris*	Autumn	Exmoor
H12	echium	*Echium plantagineum*	Summer	Warwickshire
H13	field and forest	Blend of moor, woodland and wild pasture flowers.	Mixed	Yorkshire
H14	hedgerow	Mixture of flowers from hedgerows, meadows, and farmland.	Mixed	Norfolk
H15	English blossom	Blend of blossoms from spring and summer	Mixed	Yorkshire
H16	apple blossom	*Malus domestica*	Spring	Norfolk
H17	wildflower	Mixture of summer wildflowers	Summer	Warwickshire
CH1	wildflower	Mixture of summer wildflowers and brambles	Summer	Oxfordshire
CH2	heather	Heather from Scottish hills and countryside	Autumn	Scotland
CH3	heather	Heather from Scottish moors	Autumn	Scotland
CH4	borage	*Borago officinalis*	Spring	England

[a] As described by honey producer or interpreted from product label.

2.1.2. Syrup samples

16 sugar syrups derived from corn ($n = 3$), rice ($n = 6$), or sugar beet ($n = 5$) were purchased from online retailers or grocery stores (Shehata et al., 2024), two of the syrups (S1, S2) did not state the plant origin so were treated as unknown samples (Table 2). Control samples were included consisting of rice syrup (R5) prepared in house as described in section 2.1.3, corn flour (C4), granulated sugar (B3), and syrup prepared from B3 with water (50/50 *w/v*) (B4). These samples were also stored at room temperature and away from light.

A subsample of each of the 17 honey samples was spiked with sugar syrup at 1%, 5%, 10% and 30% (*w/w*) prior to DNA extraction.

2.1.3. Rice syrup preparation

To prepare sample R5, 450 g of long grain brown rice (Sainsbury's, UK) was washed and steamed in a rice cooker (Breville ITP181 1.8 L, UK). 1L of water was added with 150 g of barley malt powder (Wang Korea, South Korea), mixed and left to ferment for 6 h at 60 °C. The mixture was strained using a muslin cloth and the liquid collected was boiled for 30 min to produce the syrup.

2.1.4. Plant material

Corn kernels (*Zea mays* NK Falkone, Syngenta, Jealott's Hill, UK), rice grains (*Oryza sativa* X265) and sugar beet seeds (*Beta vulgaris* var. Altissima, J&L seeds, France) were sterilised in 5% sodium hypochlorite: water (*v/v*) (Merck, Germany) for 20 min and washed with dd H_2O before leaving to germinate. Fresh leaf material was then taken from young plantlets for DNA extraction of plant control samples.

2.2. DNA extraction

2.2.1. DNA extraction from plant material

Plant material was snap-frozen in liquid nitrogen and ground with a 3 mm tungsten carbide bead (TC, Qiagen, Germany) in microtubes using the Star-Beater (VWR, UK) at 30 mHz for 2 min. The E.Z.N.A Plant DNA kit (Omega Bio-Tek, USA) was used for the extraction following the manufacturer's protocol. The eluted samples were stored at −20 °C, and diluted 10X in ultrapure water for PCR testing.

Table 2
Sugar syrup and control sample information.

Sample #	Description	Plant species	Production country
C1	corn syrup	corn	Korea
C2	corn syrup	corn	Italy
C3	corn syrup	corn	Korea
C4[b]	corn flour	corn	NK[a]
R1	rice syrup	rice	Spain
R2	rice syrup	rice	Spain
R3	rice syrup	rice	Korea
R4	rice syrup	rice	Korea
R5[b]	rice syrup	rice	NK[a]
R6	rice syrup	rice	Germany
R7	rice syrup	rice	Non-EU
S1	sugar syrup	NK[a]	Korea
S2	sugar syrup	NK[a]	Korea
B1	sugar beet molasses	sugar beet	Germany
B2	bee feed syrup	sugar beet	Germany
B3[b]	granulated sugar	sugar beet	UK
B4[b]	sugar syrup	sugar beet	UK
B5	light syrup	sugar beet	Sweden
B6	golden syrup	sugar beet	UK
B7	dark syrup	sugar beet and cane	Sweden

[a] NK = Not known.
[b] Control sample.

2.2.2. DNA extraction from honey/syrup

10 g pure honey, syrup, or spiked honey was diluted to 50 mL with ultrapure water and incubated at 65 °C for 30 min until completely dissolved. After centrifugation at 4500 RPM for 15 min the pellet was processed with two different extraction kit methods.

For the Nucleospin Food kit (Macherey-Nagel, Germany), the pellet was suspended in 200 μL ultrapure water, and ground with a 3 mm TC bead in the Star-Beater at 25 mHz for 2 min. After the addition of 400 μL of CF lysis buffer and 10 μL of proteinase K (20 mg/mL, QIAGEN, Germany) the samples were incubated for 30 min at 65 °C, before adding 10 μL of RNase A (10 mg/mL, ThermoFisher Scientific, UK). After this the manufacturer's protocol was followed for the DNA extraction.

For the DNeasy Plant Pro kit (QIAGEN, Germany), the pellet was suspended in 500 μL CD1 buffer and ground with a 3 mm TC bead in the Star-Beater at 25 mHz for 2 min. The samples were incubated at 65 °C for 30 min after the addition of 10 μL proteinase K (20 mg/mL). After this the manufacturer's protocol was followed.

The DNA was eluted in 50 μL elution buffer with both methods, passed twice through the column and stored at −20 °C.

2.3. DNA markers

Investigated DNA sequences were obtained from the NIH GenBank database (Benson et al., 2005), and candidate gene regions were identified for rice (*Oryza sativa*), corn (*Zea mays)* and sugar beet (*Beta vulgaris* subsp. *vulgaris*) for each species. Primers were designed to be species specific and tested firstly *in silico* by using Primer-BLAST (Ye et al., 2012), and then by using PCR (section 2.4.) to check for amplification or cross amplification in the rice, corn and sugar beet plant extracts. All primers were developed during this study, except where reference is provided (Table 3). The conserved plant marker targeting the *trnL* P6 loop was used as a general positive control (Taberlet et al., 2007).

2.4. PCR and qPCR tests

2.4.1. PCR protocol

The PCR was performed in a 10 μL reaction using 10X DreamTaq Buffer (ThermoFisher Scientific, UK), consisting of 0.2 mM dNTPs, 2 mM $MgCl_2$, 1.25 U Dream Taq DNA polymerase, 0.5 μM forward and reverse primer (Life Technologies Ltd, UK) and 1 μL DNA extraction or water for negative control. The thermocycler (PTC-200, BioRad, UK) was run at 95 °C for 3 min, then 95 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s for 35 cycles, the final extension was 72 °C for 5 min.

The products were run on a 2% TBE (Tris-Borate-EDTA, Duchefa, The Netherlands) agarose (Thermo Scientific, UK) gel at 100 V for 45 min and stained with SafeView dye (NBS Biologicals Ltd., UK) with the GeneRuler DNA ladder (ThermoFisher Scientific, UK) for size comparison (Syngene gel-doc system).

2.4.2. qPCR protocol

For qPCR a 10 μL final reaction volume was generated using SsoAdvanced Universal SYBR Green Supermix (BioRad, UK) containing antibody-mediated hot-start Sso7d fusion polymerase, dNTPs, $MgCl_2$ and SYBR Green I dye, with 0.5 μM forward and reverse primers and 1 μL DNA extract or water. The reaction was run on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, UK) at 95 °C for 3 min, then 95 °C for 15 s and 65 °C for 30 s for 40 cycles, a melt curve was produced at the end of each run from 65 °C to 95 °C at 0.5 °C increments.

2.4.3. qPCR analysis

The results were assessed either by using the raw C_q value calculated by CFX (CFX Maestro Version 2.3) applying a single threshold with baseline subtracted curve fit, or by scaling the data to set the amplification for the marker of interest at 100% for the target plant. This was done by the following equation:

Table 3
Primer information.

Target species	Target gene	Name	Sequence 5′-3′	Amplicon length (bp)
plant	tRNA-Leu (trnL) P6 loop (Taberlet et al., 2007)	*trnL_P6_f*	GGGCAATCCTGAGCCAA	10 – 144
		trnL_P6_r	CCATTGAGTCTCTGCACCTATC	
rice (*Oryza sativa*)	phospholipaseD1 (PLD1) (JRC, 2006)	*rice_PLD1_f*	TGGTGAGCGTTTTGCAGTCT	68
		rice_PLD1_r	CTGATCCACTAGCAGGAGGTCC	
	cytosinemethyltransferase Zmet3	*rice_Zmet3_f*	CCCGGCCCTAAGGAACATAG	210
		rice_Zmet3_r	CAGCAACCAAAGCACCTGAC	
	region of chloroplast	*rice_cp_f*	TTACACCGCACACCCCTG	99
		rice_cp_r	AGACCCGTTCCTTTTGGTATC	
	26S rRNA	*rice_rrn26_f*	TTTGGCGACCAGCAAACTCT	54
		rice_rrn26_r	TCATCCCTTGCTTCAAAACTAC	
corn(*Zea mays*)	formin-like protein 7 (LOC103635325) (Truong et al., 2022)	*corn_PERK2_f*	TCTATAAGCTTTGATTCCAGGG	275
		corn_PERK2_r	TTGCTCTCCGAAATGACACTAT	
	tRNA-Leu (trnL)	*corn_trnL_f*	ACTACCCCTCCCCATTTCCT	108
		corn_trnL_r	CCCTTTCTTGTGCATCATCTTA	
	26S rRNA	*corn_rrn26_f*	TCTTTGGCGACCAGCAAAGC	66
		corn_rrn26_r	CTCATCCCTTGCTTCAAAACGAA	
sugar beet(*Beta vulgaris*subsp. *vulgaris*var. *altissima*)	adenylate transporter (*ant*) (Chaouachi et al., 2013)	*beet_ant_f*	CAGTATATTTTAGTCAATTCCAAG	135
		beet_ant_r	ACATTTTCTGTCTGGTCTACTACC	
	unknown region	*beet01_f*	GCCCCCAAAAACCCTTCA	116
		beet01_r	GGGCAATTTGGTAGGCTTCTT	
		beet02_f	ATCCCTGCAGCCATCAGTGA	121
		beet02_r	ACCAGTAAGCCACTCAACAGTCAA	
	maturase K (*matK*)	*beet_matk_f*	CCCAGACCGACTTACTAACG	76
		beet_matk_r	ATTCCTCTGGTTGGATCCTTG	
	open readingframe 409 (*orf*409)	*beet_orf409_f*	GATCTTCCCAACCCATATGGA	56
		beet_orf409_r	GGCGAATAAAGTCCGACAGTA	

$$Scaled Amplification\,(\%) = (No.\,cycles - C_{q(sample)})\,x\left(\frac{100}{(No.\,cycles - C_{q(control)})}\right)$$

Where:

No. cycles is the number of cycles the qPCR program.

$C_{q(sample)}$ is the C_q value obtained in the sample for the marker of interest.

$C_{q(control)}$ is the C_q value obtained in the target plant control for the marker of interest.

For all results the average C_q and standard deviation (SD) was calculated from three replicate qPCR reactions. Negative controls were included in all tests, for the *trnL_P6* amplification was sometimes seen above 35 cycles due to the high sensitivity of the marker to all plant DNA, this is respectively shown in the results tables found in Appendix A.

2.5. Sugar composition analysis

2.5.1. Chemicals

Methanol (LC-MS grade) and acetonitrile (≥ 99.9%) were obtained from Fisher Scientific, UK. D-(−)-Fructose (> 99%), D-(+)-Glucose (> 99.5%), Sucrose (≥ 99.5%) and Maltose (> 95%) were sourced from Sigma Aldrich, UK.

2.5.2. HPLC-ELSD analysis

Honey samples (Table 1), sugar syrup samples (Table 2) and honey spiked with 10% rice syrup 1 (R1) were prepared and analysed according to the Harmonised Methods of International Honey Commission with some modifications (Bogdanov, 2009) and as described in Shehata et al. (2024).

2.5.3. Statistical analysis

The Welch Two Sample *t*-test was applied on the individual sugar content to compare pure vs rice syrup adulterated honey. The analysis was performed in R v4.4.0.

3. Results

3.1. Primer amplification and specificity

PCR reactions were performed for all 13 primer pairs, amplifying regions of nDNA, mtDNA or cpDNA of the rice, corn and sugar beet DNA extracts (Table A.1). The plant positive control marker (*trnL_P6*) was amplified in all three of the plant extracts. Of the rice markers the *PLD_1* was amplified in both rice and corn, but the *Zmet3*, *cp* and *rrn26* were specific to the rice extract. All three of the corn markers were species-specific to corn. For sugar beet the *ant* marker was not successfully amplified, while *01*, *02*, *matk* and *orf409* were only amplified in the sugar beet extract.

The amplification success of the markers was then compared using qPCR, where a lower C_q value indicated a higher amount of target was present in the sample (Table 4). These results confirmed that the *rice_PLD1* was amplified in both rice and corn, with sugar beet also amplifying *rice_PLD1* after 35 cycles, explaining why it didn't show up in the PCR. Similarly, *corn_trnL* showed limited amplification in rice (C_q

Table 4
Amplification of DNA markers in rice, corn and sugar beet plant controls using qPCR.

Primer	Target genome	Amplification (C_q)		
		rice	corn	sugar beet
trnL_P6	cp	15.11	15.01	15.17
rice_PLD1	n	23.04	26.40	35.97
rice_Zmet3	n	22.30	[a]	[a]
rice_cp	cp	16.48	38.42	38.59
rice_rrn26	mt	18.88	[a]	[a]
corn_PERK2	n	[a]	25.64	[a]
corn_trnL	cp	36.60	14.43	[a]
corn_rrn26	mt	[a]	19.17	[a]
beet_ant	n	[a]	[a]	[a]
beet01	u	[a]	[a]	24.62
beet02	u	[a]	[a]	28.54
beet_matk	cp	[a]	[a]	15.45
beet_orf409	mt	[a]	[a]	18.46

[a] Not amplified, cp: chloroplast, mt: mitochondria, n: nuclear, u: unknown target.

36.60) and *rice_cp* showed low amplification in both corn and sugar beet extracts (C_q 38.42 and 38.59). All other markers were only amplified in the species of interest, apart from the *beet_ant* marker which failed to amplify. In general, the specific markers targeting nuclear genes (nDNA) produced results with higher C_q values for the target species (C_q 22.30–25.64), the mitochondrial markers (mtDNA) produced C_q values of 18.46–19.17 and the chloroplast markers (cpDNA) produced the lowest C_q values between 14.43 and 16.48 for the target species. However, the chloroplast markers for rice and corn showed a lower specificity, suggesting that the mitochondrial markers might be a better choice for species specificity.

3.2. DNA extraction from sugar syrups

3.2.1. Amplification of general plant marker

The two DNA kits QIAGEN DNeasy Plant Pro and Macherey-Nagel Nucleospin Food Kit were used to extract plant DNA from the sugar syrups, and extraction efficiency was evaluated by assessing the amplification of the chloroplast *trnL* P6 loop with qPCR to evaluate the yield of plant DNA (Table A.2). For both kits the DNA extraction was successful, with the *trnL_P6* marker displaying amplification above 32 cycles in all samples, which is notable given the difficult sample matrix. For all samples the C_q value was lower for the Nucleospin Food kit, indicating a higher plant DNA yield and more efficient extraction capacity with this kit. Samples C4 and B4-7 were not extracted using the Plant Pro kit therefore not included in this analysis. The Nucleospin Food kit was subsequently used for further extractions in this study.

3.2.2. Amplification of plant specific markers

Based on the specific marker amplification results, one marker was selected for each species: *rice_rrn26*, *corn_rrn26* and *beet_matk* for rice, corn and sugar beet respectively and all syrup DNA extracts were evaluated for marker specificity by qPCR.

The results, shown in Fig. 1, are scaled to the target plant DNA amplification as 100%. The C_q and SD values are shown in Table A.3, including the *trnL_P6* plant marker results for comparison. All rice syrups showed amplification for the *rice_rrn26* marker, ranging from C_q 19.73 (R5) to C_q 37.32 (R7). Low amplification was seen in other sugar syrups (C1, C2, C3, S1 and S2), but with high C_q values ranging from 35.33 to 37.59. The *corn_rrn26* marker was amplified in one out of three corn syrups (C2), as C4 was the control sample. Amplification for the *corn_rrn26* marker was also seen in R4 (C_q 37.11), R5 (C_q 31.58), S1 (C_q 37.28) and B3 (C_q 35.94). The *beet_matK* marker did not show strong amplification in any sample other than the sugar beet plant extract. The sugar beet syrup extracts showed C_q values between 33.44 and 35.68 for *beet_matK*, with no amplification in B4, B5 and B7. Similar amplification values were seen for *beet_matK* across the other sugar syrups (C_q 33.48–36.69), leading to the conclusion that this is unlikely to be linked to the presence of sugar beet DNA in the samples.

Therefore, no further work was carried out with the *beet_matK* marker. All three of the other successful beet markers (*beet01, beet02* and *beet_orf409)* were also tried with the sugar beet syrup extracts, with no amplification success (results not shown).

3.3. Detection of rice and corn markers in adulterated honey samples

To reproduce adulterated honey rice syrups R1, R2 and R4, and corn syrup C2 were selected to spike natural honey H17. Both the rice and corn marker were detected in 1% spiked honey (Fig. 2) with C_q values proportionate to the percentage spike, except in the case of R1 where the 5% spiked sample had a lower value (C_q 25.09) than the 30% (C_q 26.84) and 10% (C_q 26.73) spike.

For both the rice and corn marker there was a low amplification in the pure honey sample (0% spike) but producing considerably higher C_q values than the 1% spike sample. Between the 1% spiked and the pure honey sample a difference of ~5 cycles with the corn marker and between ~8 and 10 cycles for the rice marker was observed, permitting the clear separation of pure honey from the lowest 1% spike.

3.4. Marker amplification in different honey types

3.4.1. Rice and corn marker background amplification

To analyse the background amplification of the rice and corn markers detected in natural honey, these markers were tested for amplification in 16 different honey types, in order to determine a threshold value for the method. Honey H15 was excluded from the results as there was no amplification with the *trnL_P6* marker used as a positive control for the plant DNA (Table A.5). The rice marker was amplified in 8 out of 16 honey samples and the corn marker in 10 out of 16 honey samples. For both markers the buckwheat honey (H8) showed the strongest amplification with C_q values at 35.89 for *rice_rrn26* and 32.67 for *corn_rrn26*, with the corn marker showing a higher presence in the samples where it showed amplification. To guarantee that the test would not flag natural honey as adulterated honey, thresholds were set for each marker by taking the sample with the lowest C_q and adding a 95% confidence interval. For rice the threshold was 34.27 cycles and for corn it was 31.27 cycles (Fig. 3).

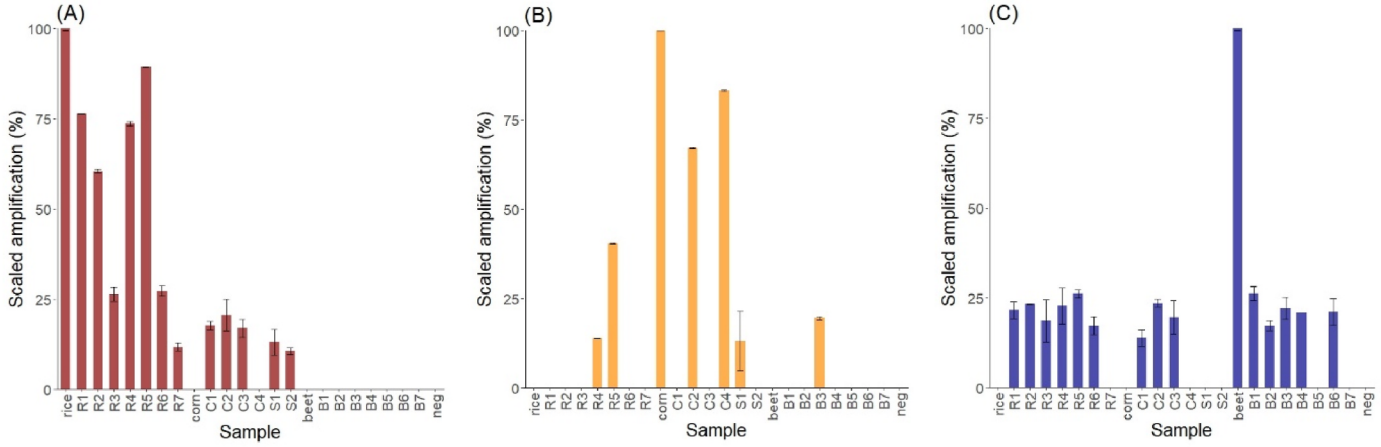


Fig. 1. Amplification of DNA markers (A) rice_rrn26, (B) corn_rrn26 and (C) beet_matK with plant and sugar syrup extracts (Table 2). Amplification scaled where amplification for the target plant is set at 100% and error bars representing the standard deviation from three PCR reactions.

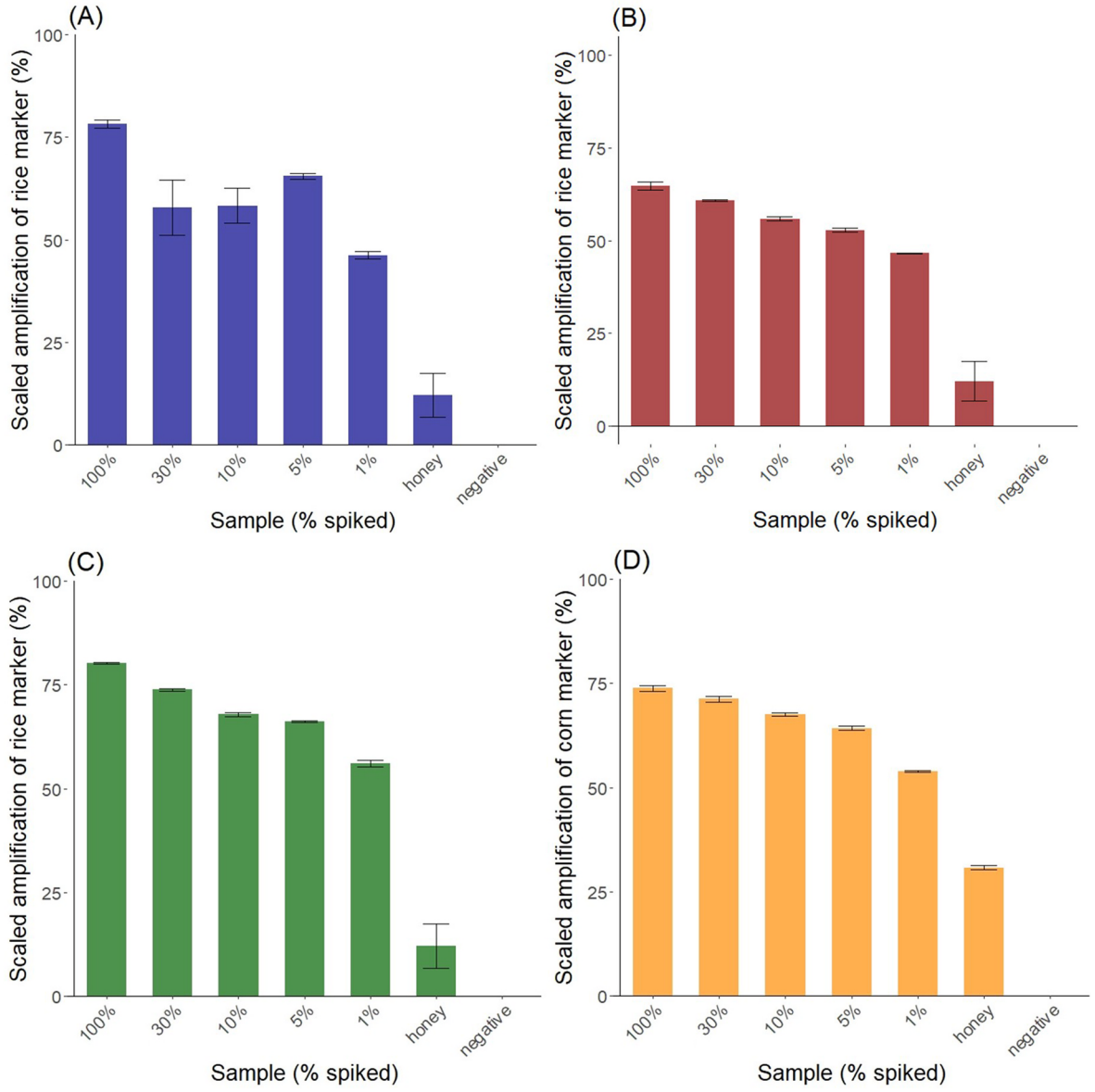


Fig. 2. Amplification of DNA markers (A–C) rice_rrn26 and (D) corn_rrn26 for honey spiked with rice syrups (A) R1, (B) R2, (C) R4 and corn syrup (D) C2. Amplification scaled where target plant amplification was set to 100% and error bars representing the standard deviation from three PCR reactions.

3.4.2. *Rice marker amplification in 5% spiked honeys*

Each honey type was spiked with 5% rice syrup R1 and tested for the *rice_rrn26* marker amplification to determine the suitability of the test for different honeys. Extracts which did not show amplification for the plant *trnL* P6 loop (H4, H10 and H15) were excluded from the analysis. All spiked honey samples showed amplification for the *rice_rrn26* marker above the threshold of 34.27 cycles (corresponding to 25.03% when scaled) as shown in Fig. 4.

3.4.3. *Application to commercial honeys*

Commercial samples marketed as UK origin honeys were purchased from the UK market and extracted with the described protocol. The honey extracts were tested for amplification of the *trnL_P6*, *rice_rrn26* and *corn_rrn26* markers (Table A.7). Three of the commercial honey extracts (CH2 - CH4) showed good amplification of plant DNA based on the *trnL* P6 marker (C_q 19.16 to 23.97), but one commercial honey extract (CH1) showed no amplification for the plant DNA marker. Of the three honeys which amplified the plant marker there was no amplification of the *rice_rrn26* marker. In the same three extracts there was low amplification of the *corn_rrn26* marker (C_q 35.67 to 38.47), similar to that seen in other UK honeys tested in this study.

3.5. *Sugar composition of pure, spiked and commercial honey samples*

Each sugar syrup, honey type, honey type spiked at 10% with R1 and commercial honey was subjected to HPLC-ELSD analysis to quantify the amount of fructose, glucose, sucrose and maltose in the sample (Table 5). Based on the obtained results and considering a 95% confidence interval, the 17 pure honey samples and 4 commercial honey samples were all below the 5 g/100g limit for sucrose. Even H07 and CH4, the borage honeys, which have an increased limit of 15 g/100g according to legislation (Codex Alimentarius, 1981; Honey (England) Regulations, 2015). However, when assessing the sum of fructose and glucose, one honey sample (H11) was under the 60 g/100g limit with a value of 56.79 g/100g, meaning it would not comply with the honey regulations. All commercial honey samples were within regulation for sugar composition. Compositional analysis of the rice syrup used for spiking (R1) showed that it was composed of 1.49 g/100g fructose,

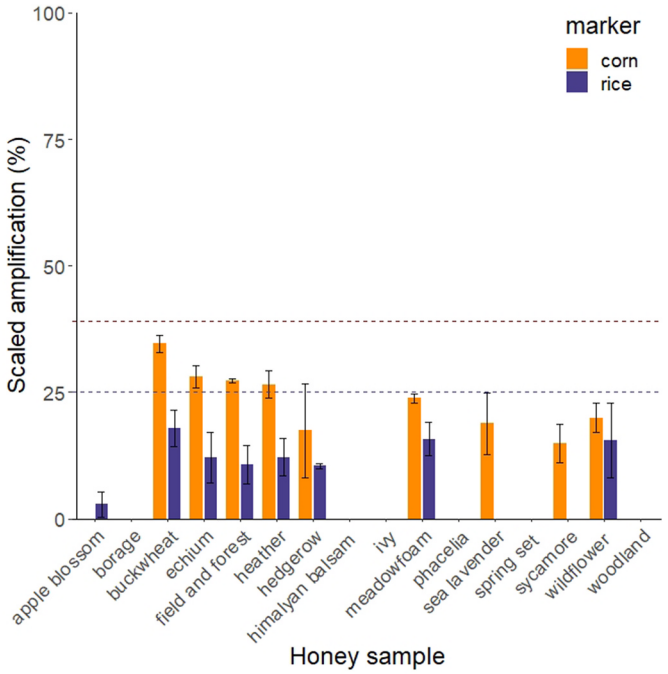


Fig. 3. Amplification of *rice_rrn26* and *corn_rrn26* in natural honey. Thresholds are shown as dashed lines. Amplification scaled where target plant amplification was set to 100% and error bars representing the standard deviation from three PCR reactions.

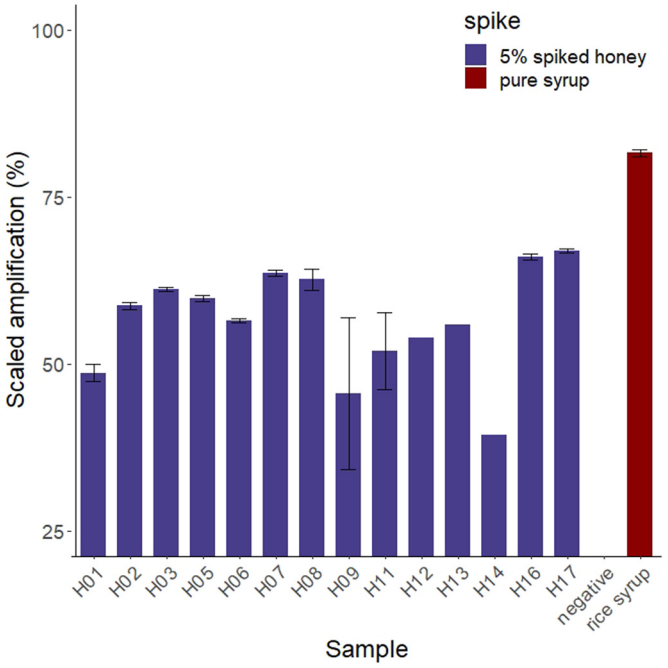


Fig. 4. Amplification of *rice_rrn26* in different honey types spiked with 5% rice syrup (R1), and pure rice syrup (R1). The y-axis is cropped to represent the rice threshold (25.03%). Amplification scaled where target plant amplification was set to 100% and error bars representing the standard deviation from three PCR replicates.

22.49 g/100g glucose, 0.22 g/100g sucrose and 31.90 g/100g of maltose, with a fructose-glucose sum of 23.99 g/100g. Therefore, it is not surprising that the sucrose level of the honeys spiked with R1 did not increase over the legislated 5 g/100g or 15 g/100g threshold. In all 17 of the honey types spiked with rice syrup, the total of fructose and glucose fell below the 60 g/100g legislation, with a mean concentration of 67.27 g/100g before spiking, and 50.57 g/100g after spiking. Consequently, these pseudo-adulterated samples would have been flagged as non-compliant of the honey standard regulations. Moreover, the maltose concentration of R1-spiked samples was significantly elevated compared to the pure honeys with mean concentrations of 1.43 g/100g and 3.47 g/100g respectively ($p < 0.01$).

Table 5
Sugar composition of honey, honey spiked with 10% rice syrup (R1) and syrup samples.

Sample	Spike (%)	Fructose (g/100g)	Glucose (g/100g)	Sucrose (g/100g)	Maltose (g/100g)	F + G (g/100g)
H01	0	36.18	29.00	0.99	2.14	65.18
	10	26.74	22.53	0.72	3.88	49.27
H02	0	38.37	30.80	1.41	0.58	69.17
	10	26.36	22.76	1.08	2.89	49.11
H03	0	38.96	31.76	1.84	2.16	70.72
	10	27.71	22.28	2.50	3.06	49.98
H04	0	33.35	38.18	0.59	1.90	71.53
	10	23.42	27.67	1.08	3.59	51.09
H05	0	37.92	28.75	0.91	0.98	66.67
	10	27.70	22.49	0.74	4.24	50.19
H06	0	38.10	35.58	0.68	0.75	73.68
	10	26.86	26.75	0.54	2.91	53.61
H07	0	37.26	31.24	4.86	2.75	68.49
	10	26.31	23.21	3.27	4.39	49.52
H08	0	35.09	35.32	1.37	0.21	70.42
	10	26.05	24.50	0.99	2.62	50.55
H09	0	37.87	35.19	0.50	1.70	73.06
	10	26.74	24.83	0.33	2.60	51.56
H10	0	34.11	26.43	0.55	1.73	60.54
	10	27.34	23.36	0.47	2.94	50.70
H11	0	33.51	23.29	1.23	0.52	56.80
	10	28.73	21.96	1.04	3.30	50.68
H12	0	32.93	26.33	0.77	1.78	59.26
	10	27.33	23.46	0.66	3.91	50.79
H13	0	33.53	26.10	0.65	0.67	59.64
	10	27.70	21.47	0.70	2.68	49.16
H14	0	33.02	26.44	1.13	0.80	59.46
	10	27.78	23.38	0.99	4.13	51.16
H15	0	41.61	33.92	0.96	1.95	75.54
	10	27.93	24.05	0.56	4.42	51.98
H16	0	38.74	31.93	1.50	0.33	70.66
	10	26.61	23.24	1.03	3.68	49.85
H17	0	40.81	32.00	4.15	3.41	72.81
	10	28.11	22.28	2.77	3.78	50.39
CH1	0	37.69	30.72	4.90	0.46	68.42
CH2	0	44.69	33.59	1.94	0.45	78.27
CH3	0	43.05	31.34	3.35	0.51	74.39
CH4	0	35.84	30.48	4.25	3.04	66.32
R1	0	1.49	22.49	0.22	31.90	23.99
R2	0	1.49	23.31	0.25	28.57	24.80
R3	0	1.68	17.34	0.85	42.59	19.02
R4	0	1.77	9.54	1.51	28.94	11.31
R5	0	1.65	9.76	0.50	12.39	11.41
R6	0	1.49	23.01	0.32	36.70	24.51
R7	0	1.79	26.90	0.62	37.14	28.69
R8	0	3.58	28.11	1.49	33.10	31.69
C1	0	1.49	0.82	0.75	41.16	2.31
C2	0	1.79	22.53	0.86	33.48	24.32
C3	0	1.49	1.56	1.93	50.86	3.05
S1	0	1.83	17.19	0.54	8.97	19.01
S2	0	5.12	23.24	18.95	0.30	28.36
B1	0	19.03	16.40	27.34	0.21	35.43
B2	0	39.71	31.03	7.92	0.30	70.74
B4	0	1.49	0.43	53.99	0.29	1.92
B5	0	24.74	24.37	31.52	0.27	49.11
B6	0	23.88	26.97	34.64	0.40	50.85
B7	0	24.13	23.67	31.64	0.28	47.80

4. Discussion

4.1. DNA markers and their specificity

In this study a DNA barcoding approach to authenticate honey was evaluated using novel endogenous markers developed for rice, corn, and sugar beet syrup detection. Typically, reference genes are required to be species-specific, having a stable low copy number with limited intra-species variability (Chaouachi et al., 2013). They are generally used to determine genetically modified organisms (GMOs) occurrence in ingredients, as the presence of the modified gene is compared to that of the reference gene (Xiujie et al., 2019). However, in the case of highly processed food products, such as sugar syrups, low copy number genes are less likely to be detected due to the low amount of degraded DNA present in the sample (Caldwell, 2017). Hence, it makes more sense to target genome regions with high copy number, such as chloroplast or mitochondrial genes, to increase the chance of detection. Although this reduces the possibility of accurate fraud quantification, in the case of honey authentication this is not a necessity, as simple identification of adulterant material can provide substantial evidence for misconduct (Nehal et al., 2021). In our study, appropriate DNA markers were chosen from literature, or designed in house, targeting nuclear, chloroplast and mitochondrial genes in rice, corn, or sugar beet.

Most of the applied markers showed flaws, such as no amplification or cross amplification with other species. The *beet_ant* marker was developed by Chaouachi et al. (2013) as a reference marker specific to sugar beet (*Beta vulgaris* L). They found the marker amplified in all 39 of the *Beta vulgaris* L varieties tested, with no amplification in closely related species. However, in our study the *beet_ant* marker failed to amplify, despite various annealing temperatures being tested. This could be due to a different variety of sugar beet being used, as the "Altissima" variety used in our lab was not included in their study, but these results unfortunately rendered the *beet_ant* primer unsuitable as an endogenous sugar beet marker. The *rice_PLD1* marker was utilised by Sobrino-Gregorio et al. (2019) to detect honey adulteration with rice molasses, they determined that it was the most effective primer used in their study providing quantification at 2 – 5% when using a standard curve of rice genomic DNA. However, we showed that the *rice_PLD1* marker was also amplified in the corn and sugar beet extracts. Moreover these results were confirmed by Xiujie et al. (2019), who found that the *rice_PLD1* displayed non-specific binding in both maize and potato, further providing evidence that this is not an accurate rice specific marker for our studies.

Of the remaining successful DNA markers, the primers targeting regions of nDNA produced results with higher C_q values than those targeting cpDNA or mtDNA. Although nuclear markers have been used with success in other studies, there are clear advantages to using higher copy number targets. For instance, Truong et al. (2022) were successfully using the nuclear *corn_PERK2* marker to detect Korean honey adulterated with corn syrup, but they were only able to reach a detection limit of $\geq$ 10%. Here we have shown that with the use of mitochondrial markers we could detect corn syrup adulterated honey at 1%, indicating that plastid markers are a more suitable choice to achieve increased sensitivity. Indeed, mitochondrial DNA is widely used in DNA analysis of food products, as it is proven that the plastid structure further reduces the DNA degradation (Sajali et al., 2018). Although the chloroplast markers used in this study produced lower C_q values (higher detection) than the mitochondrial markers, they had a lower specificity to the target plant, as the corn and rice cpDNA markers showed cross amplification with other non-target plant DNA extracts. This is due to the fact that chloroplast genomes are more conserved than the mitochondria genomes, possessing a slower rate of evolution and sequence variability (Li et al., 2022). This can also explain why the *beet_matK* chloroplast marker showed marginal amplification levels in many of the sugar syrup extracts, despite appearing species specific when tested on the rice and corn plant DNA.

4.2. DNA extraction from sugar syrups

One of the challenges of DNA based food authentication tests is the DNA extraction, as food products contain a complex matrix of ingredients which may have been altered by different processing types. Honey itself is a difficult matrix for DNA extraction due to the presence of various PCR inhibitors such as organic acids, polyphenols, pigments, enzymes, and wax particles. However, numerous studies have shown success with commercial kits aiming for DNA isolation from plants or food products (Chiara et al., 2021; Hawkins et al., 2015; Khansaritoreh et al., 2020; Soares et al., 2018; Wu et al., 2017). DNA extraction from sugar syrups have a further issue of being highly processed causing high DNA fragmentation and reducing the quantity of target DNA in the sample. Furthermore, if they are added to honey samples only in small amounts, it will decrease even more the residual syrup DNA content in the sample. Another challenge is how to assess the quality and quantity of DNA extracted from the sample, as there can be multiple types of DNA (plant, animal, microbial) present there. This implies that typical spectrophotometer measurements (such as nanodrop data) will represent the total DNA content of the sample, as opposed to just the DNA type of interest, hence this can be an inaccurate way to assess the success of expected food DNA extractions. We used a conserved plant marker as a mean of evaluating the DNA extractions in terms of plant DNA yield. The primer targeting the *trnL* P6 loop was shown to perform excellently on highly degraded DNA, due to its short amplification product (10 – 143 bp) and high copy number. It displayed 100% amplification success in a food dataset including rice, corn and beet, making it a highly appropriate marker for this study (Taberlet et al., 2007). Moreover, the *trnL* P6 marker has already been used in studies exploring the origin of plants in honey samples with metabarcoding of the region, but has yet to be applied to sugar syrup adulteration detection (Chiara et al., 2021; Valentini et al., 2010).

We found that the Nucleospin Food kit produced a higher yield of plant DNA from syrup extracts compared to the DNeasy Plant Pro kit. Similarly, Sobrino-Gregorio et al. (2019) found that the NucleoSpin Food kit provides high quality DNA extractions from rice molasses. Although the DNA extraction method proved to be successful for all the sugar syrup samples, in the case of one pure honey sample (H15), one commercial honey sample (CH1) and three of the 5% rice syrup spiked honey samples (H04, H10 and H15) there was no amplification with the *trnL_P6*, suggesting that no plant DNA was present in the sample, or the extraction protocol was not suitable for these samples (extraction repeated three times with no success). For this reason, these samples were excluded from the analysis as a more optimised DNA extraction may be required to remove PCR inhibitors or increase the DNA yield in these samples.

4.3. DNA markers in syrup extracts

The amplification of the specific DNA markers varied greatly amongst the different types of rice and corn syrups, with samples amplifying the target marker weakly (R7) or not at all (C1 and C3). For syrups R1, R2, R4, R5 and C2 the amplification of the *trnL_P6* plant marker and the target rice or corn marker had similar C_q values, amplifying within 3 cycles of each other, suggesting that rice or corn respectively was a main component of the syrup. But there were some samples with inconsistencies between the amplification of the plant marker and the species-specific marker, for example in R3 the *trnL_P6* amplified with a C_q of 26.42, but the *rice_rrn26* marker amplified later with a C_q of 34.02. Similar patterns were seen with R6 (C_q 27.81 and 33.8) and R7 (C_q 26.78 and 37.32), suggesting that the quantity of rice target in the sample was much less than the total amount of plant DNA. Although this could be because *rice_rrn26* primer target sequence had undergone degradation in the sample, it could also indicate that the syrups themselves are not authentic. However, it could also be that different rice varieties were used to prepare the syrup, possessing

sequence variations at the primer target site as the primer specificity tests were only performed with one rice line. This inconsistent pattern was also seen with the corn and beet syrup extracts which did not amplify the target marker, while there was undoubtedly plant DNA in the sample due to the positive *trnL_P6* amplification, nonetheless this could not be attributed to corn or sugar beet DNA using the qPCR test.

We were unable to achieve sufficient amplification of the sugar beet markers in the sugar beet syrup extracts, even though the used markers had shown significant amplification in the sugar beet plant control sample. Similar results have been obtained for sugar products derived from sugar beet, where Oguchi et al. (2009) suggested that sugar beet DNA had degraded during the early stage of sugar processing, as specific markers *beet01* and *beet02* (included in this study) were amplified in sugar beet juice, but not in subsequent sugar products. However, limited success was produced with sugar cane products in a study by Wang et al. (2020), who found that DNA was present in low amounts in extracts prepared from sugar cane molasses, refined syrup and crystalised sugar samples, with PCR amplification in all but one sugar extracts. Thus, the positive amplification obtained for the *trnL_P6* for corn syrups C1 and C3, and all sugar beet syrups should be further investigated to confirm the success of the extraction protocol, which could be assisted by amplicon sequencing of this marker. This method could also be used to identify the plant origin of the unknown syrup samples (S1 and S2), as either the rice, corn or sugar beet markers were strongly amplified in these samples.

4.4. Amplification of rice and corn markers in natural and adulterated honey samples

The *rice_rrn26* and *corn_rrn26* markers were tested for amplification in syrup spiked honey samples and were positively amplified at all concentrations tested including at 1% adulteration level. The sensitivity of this test is higher than other studies obtained with similar methods: 2 – 5% and $\geq$ 10% for rice and corn respectively (Sobrino-Gregorio et al., 2019; Truong et al., 2022), which is likely due to the specific plastid markers used in our work. Low marker amplification was seen in the pure honey, but it is reasonable to expect that rice or corn DNA could be present in small amounts in natural honey due to bee foraging or leftover winter bee feed making its way into the honey. In the UK, corn is a prevalent crop which, although does not produce nectar and therefore is not a honey-attributing plant, is rich in pollen, which the bees may use as a protein source (Becher et al., 2016; Höcherl et al., 2012). This could explain why the corn marker was amplified stronger in the natural honey than the rice marker, as rice is not grown in the UK.

For this reason, we tested different UK honey types to assess the expected background amplification of the *rice_rrn26* and *corn_rrn26* markers in natural honey. These results were used to create a threshold where the sample is identified as adulterated or natural. When the threshold was applied to the same honey types spiked with 5% rice syrup (R1), 100% of samples were classified correctly as adulterated, proving the success of the method for this rice syrup type. However, this has not yet been tried with corn syrups or other types of rice syrups. If the syrup solely displays a low amplification of target DNA it may not pass the threshold, as it was the case of R7 where even the pure syrup would not be flagged as an adulterant based on the amplification of the *rice_rrn26* marker. Additionally, the thresholds developed for this study were calculated using a small sample set of 17 honeys. So, further work may be needed to build up a database of reference honeys to generate a greater understanding of the expected background amplification. Therefore, the right threshold with appropriate control amplifications is vital to prevent the natural honey being wrongly classified as adulterated. Besides, the honey types tested in this study were premium honeys obtained from UK beekeepers, therefore it was not an accurate representation of the UK honey market - which consists mostly of imported honey blends. However, the honeys used in this study were an appropriate representation of the many different floral sources present in the UK over multiple seasons, therefore a good interpretation of potential non-specific amplification of the markers was permitted with the natural plant sources in UK honey. The method should next be tested on honeys from different countries, and honeys which are more representative of the UK honey market. Furthermore, samples should be taken from countries where rice has a major part in the agricultural system, to determine if the threshold developed in this study is suitable for non-UK honeys.

4.5. Commercial samples

The proposed qPCR method was tested on commercial samples of UK origin purchased from the UK market, the samples were marketed as English (CH1, CH4) or Scottish (CH2, CH3) and therefore were suitable for the test as developed for UK origin honeys. The DNA extraction method was suitable for 3 out of 4 of the selected commercial honey samples, with the three successful samples showing good amplification of the plant DNA marker. For one sample (CH1) there was no amplification of the plant marker used as a positive control, suggesting that the extraction protocol was not suitable for this honey. This sample was therefore not analysed for amplification of the rice or corn marker. The rice marker was not amplified in any of the commercial honey samples, suggesting that no rice DNA was present in these honeys. For the corn marker there was low amplification in all three samples, comparable to results seen in the other UK honeys and therefore under the natural threshold developed for this study. This aligned with the principle that corn may be prevalent in UK honey types and should be something to be expected from commercial samples where corn is grown in the agricultural system in the country of origin. However, these limitations can be accounted for by developing representative thresholds such as displayed in this study. Applying the method to commercial honey samples highlighted the importance of including the *trnL* P6 plant control marker to check the presence of plant DNA in the extract, to verify whether the negative amplification of the adulterant marker was truly negative or just due to an insufficient DNA extraction.

4.6. Combination with traditional methods

Typically, for honey authentication, a weight of evidence or evaluative reporting approach should be taken, given the known complexities of honey analysis (Walker et al., 2022). This implies that a single atypical result should trigger further investigations, with multiple different analyses being used to add to the elements of evidence before a final decision about product non-compliance is made. For this reason, we used traditional sugar composition analysis (HPLC) alongside the DNA results. Of the pure honeys, only one sample (H11, heather honey) was flagged as non-compliant by HPLC for having a sugar (fructose + glucose) content of lower than 60 g/100g, which would trigger further analyses. However, this failure was marginal at only 0.36 g/100g under the limit when taking the CI into account. The DNA results for the rice and corn marker in this sample were negative, providing contrasting evidence that the sample was not adulterated with rice or corn syrup. In this case, the sample could be subjected to further tests to confirm the purity of the honey sample, as the dual analyses were not in agreement. The HPLC results for the commercial honey samples were compliant with legislation, which was in line with the DNA analysis suggesting that the samples were not suspicious of adulteration with corn or rice syrup. The rice syrup spiked honey samples were all flagged as non-compliant for total fructose and glucose content using HPLC. This was further confirmed by the DNA results, showing amplification of the rice marker over the natural threshold in UK honey (Fig. 4). In addition, the maltose concentrations for the 10% spiked honey samples were elevated compared to the pure honeys, which could also be taken into account despite no official limits currently existing for maltose in honey. Hence, for the adulterated samples there would be a growing weight of evidence suggesting non-compliance by the outcomes from these two analyses.

Furthermore, the DNA results could be used to determine the type of syrup added, which could assist in deciding which further tests to carry out, e.g. for presence of the AFGP rice marker. The proposed DNA method has great potential to be used alongside traditional honey analysis, modern analytical methodologies, and rapid spectroscopic screening tests as a highly sensitive method to identify the species origin of certain plant-based syrups and help confirm fraudulent practices.

5. Conclusions

A specialised method was developed to detect rice and corn syrup adulteration in honey using specific DNA markers with qPCR. The method was suitable to detect 1% honey adulteration, and a threshold was set to prevent false positive results. Moreover, different honey and syrup types were evaluated with the syrup type identified as a major factor to the success of the method, where sugar beet syrups were failing to amplify with the specific marker. Yet, the method was effective on most honey types and commercial samples of UK origin, overcoming the pitfalls of many honey authentication tests which are impacted by the large variation of honey compositions. However, the method should be assessed on a wide range of commercially relevant honey and syrup types from different countries to ensure the marker specificity and calculate the appropriate fraudulent thresholds. Furthermore, the sugar composition of the honey samples was used to detect non-specific adulteration in spiked samples, with amplification of the specific DNA marker confirming adulteration with rice syrup. Therefore, the methodology could be used as a robust test alongside current honey screening methods to help confirm product authenticity and identify the plant origins of adulterant material by applying specific markers. Nonetheless, it is vital that the results from multiple honey analyses are considered during method validation to reduce the occurrence of false positives and false negatives.

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CRediT authorship contribution statement

Sophie Dodd: Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Zoltan Kevei:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Zahra Karimi:** Writing – review & editing, Investigation. **Bhavna Parmar:** Writing – review & editing. **David Franklin:** Writing – review & editing. **Anastasios Koidis:** Writing – review & editing. **Maria Anastasiadi:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

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

Data supporting this study are included within the article and/or supporting materials. The underlying data can be accessed at https://doi.org/10.57996/cran.ceres-2589.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2024.110772.

References

Becher, M. A., Grimm, V., Knapp, J., Horn, J., Twiston-Davies, G., & Osborne, J. L. (2016). BEESCOUT: A model of bee scouting behaviour and a software tool for characterizing nectar/pollen landscapes for BEEHAVE. *Ecological Modelling, 340*, 126–133. https://doi.org/10.1016/j.ecolmodel.2016.09.013

Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Wheeler, D. L. (2005). GenBank. *Nucleic Acids Research, 33*(DATABASE ISS), D34–D38. https://doi.org/10.1093/nar/gki063. Scopus.

Bogdanov, S. (2009). Harmonised methods of the international honey commission. International Honey Commision. Available online: https://ihc-platform.net/ihcmethods2009.pdf [accessed 25 January 2024].

Caldwell, J. M. (2017). Food analysis using organelle DNA and the effects of processing on assays. *Annual Review of Food Science and Technology, 8*(1), 57–74. https://doi.org/10.1146/annurev-food-030216-030216

Chaouachi, M., Alaya, A., Ali, I. B. H., Hafsa, A. B., Nabi, N., Bérard, A., Romaniuk, M., Skhiri, F., & Saïd, K. (2013). Development of real-time PCR method for the detection and the quantification of a new endogenous reference gene in sugar beet "Beta vulgaris L.": GMO application. *Plant Cell Reports, 32*(1), 117–128. https://doi.org/10.1007/s00299-012-1346-5

Chiara, B., Francesco, C., Fulvio, B., Paola, M., Annalisa, G., Stefania, S., Luigi, A. P., & Simone, P. (2021). Exploring the botanical composition of polyfloral and monofloral honeys through DNA metabarcoding. *Food Control, 128*, Article 108175. https://doi.org/10.1016/J.FOODCONT.2021.108175

Codex Alimentarius. (1981). *Standard for honey CXS 12-1981*. International Food Standards.

Cordella, C., Militão, J. S. L. T., Clément, M.-C., Drajnudel, P., & Cabrol-Bass, D. (2005). Detection and quantification of honey adulteration via direct incorporation of sugar syrups or bee-feeding: Preliminary study using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and chemometrics. *Analytica Chimica Acta, 531*(2), 239–248. https://doi.org/10.1016/j.aca.2004.10.018

da Silva, P. M., Gauche, C., Gonzaga, L. V., Costa, A. C. O., & Fett, R. (2016). Honey: Chemical composition, stability and authenticity. *Food Chemistry, 196*, 309–323. https://doi.org/10.1016/j.foodchem.2015.09.051

EU. (2002). COUNCIL DIRECTIVE 2001/110/EC of 20 December 2001 relating to honey. *Official Journal of the European Communities, L 10/47*, 1–6.

Fakhlaei, R., Selamat, J., Khatib, A., Razis, A. F. A., Sukor, R., Ahmad, S., & Babadi, A. A. (2020). The toxic impact of honey adulteration: A review. *Foods, 9*(11). https://doi.org/10.3390/foods9111538. Article 11.

García, N. L. (2018). The current situation on the international honey market. *Bee World, 95*(3), 89–94. https://doi.org/10.1080/0005772X.2018.1483814

Guler, A., Kocaokutgen, H., Garipoglu, A. V., Onder, H., Ekinci, D., & Biyik, S. (2014). Detection of adulterated honey produced by honeybee (Apis mellifera L.) colonies fed with different levels of commercial industrial sugar (C_3 and C_4 plants) syrups by the carbon isotope ratio analysis. *Food Chemistry, 155*, 155–160. https://doi.org/10.1016/j.foodchem.2014.01.033

Hawkins, J., de Vere, N., Griffith, A., Ford, C. R., Allainguillaume, J., Hegarty, M. J., Baillie, L., & Adams-Groom, B. (2015). Using DNA metabarcoding to identify the floral composition of honey: A new tool for investigating honey bee foraging preferences. *PLoS One, 10*(8), Article e0134735. https://doi.org/10.1371/journal.pone.0134735

HMRC. (2023). UK trade info, overseas trade data: HS-0409 natural honey. https://www.uktradeinfo.com/trade-data/ots-custom-table/.

Höcherl, N., Siede, R., Illies, I., Gätschenberger, H., & Tautz, J. (2012). Evaluation of the nutritive value of maize for honey bees. *Journal of Insect Physiology, 58*(2), 278–285. https://doi.org/10.1016/j.jinsphys.2011.12.001

Khansaritoreh, E., Salmaki, Y., Ramezani, E., Azirani, T. A., Keller, A., Neumann, K., Alizadeh, K., Zarre, S., Beckh, G., & Behling, H. (2020). Employing DNA metabarcoding to determine the geographical origin of honey. *Heliyon, 6*(11), Article e05596. https://doi.org/10.1016/j.heliyon.2020.e05596

Laube, I., Hird, H., Brodmann, P., Ullmann, S., Schöne-Michling, M., Chisholm, J., & Broll, H. (2010). Development of primer and probe sets for the detection of plant species in honey. *Food Chemistry, 118*(4), 979–986. https://doi.org/10.1016/J.FOODCHEM.2008.09.063

Lazarević, K. B., Jovetić, M. S., & Tešić, Ž. L. (2017). Physicochemical parameters as a tool for the assessment of origin of honey. *Journal of AOAC International, 100*(4), 840–851. https://doi.org/10.5740/jaoacint.17-0143

Li, F., Liu, Y., Wang, J., Xin, P., Zhang, J., Zhao, K., Zhang, M., Yun, H., & Ma, W. (2022). Comparative analysis of chloroplast genome structure and phylogenetic relationships among six taxa within the genus catalpa (bignoniaceae). *Frontiers in Genetics, 13*. https://www.frontiersin.org/articles/10.3389/fgene.2022.845619.

Lopes, A. R., Moura, B. M. V., M, Grazina, L., Costa, J., Amaral, J. S., Alice Pinto, M., & Mafra, I. (2023). Authentication of incense (Pittosporum undulatum Vent.) honey from the Azores (Mel dos Açores) by a novel real-time PCR approach. *Food Chemistry, 411*, Article 135492. https://doi.org/10.1016/j.foodchem.2023.135492

Madesis, P., Ganopoulos, I., Sakaridis, I., Argiriou, A., & Tsaftaris, A. (2014). Advances of DNA-based methods for tracing the botanical origin of food products. *Food Research International, 60*, 163–172. https://doi.org/10.1016/j.foodres.2013.10.042

McDonald, C. M., Keeling, S. E., Brewer, M. J., & Hathaway, S. C. (2018). Using chemical and DNA marker analysis to authenticate a high-value food, manuka honey. *Npj Science of Food 2018, 2*(1), 1–14. https://doi.org/10.1038/s41538-018-0016-6, 2:1.

Misslera, J., Wiezoreka, T., & Beckha, G. (2016). Mannose: A marker for adulteration with syrup or resin treatment of blossom honey. *MRFS, 16*, 17–20. https://doi.org/10.1255/mrfs.4

Montilla, A., Ruiz-Matute, A. I., Sanz, M. L., Martínez-Castro, I., & del Castillo, M. D. (2006). Difructose anhydrides as quality markers of honey and coffee. *Food Research International, 39*(7), 801–806. https://doi.org/10.1016/j.foodres.2006.03.002

Morales, V., Corzo, N., & Sanz, M. L. (2008). HPAEC-PAD oligosaccharide analysis to detect adulterations of honey with sugar syrups. *Food Chemistry, 107*(2), 922–928. https://doi.org/10.1016/j.foodchem.2007.08.050

Nehal, N., Choudhary, B., Nagpure, A., & Gupta, R. K. (2021). DNA barcoding: A modern age tool for detection of adulteration in food. *Critical Reviews in Biotechnology, 41*(5), 767–791. https://doi.org/10.1080/07388551.2021.1874279

Oguchi, T., Onishi, M., Chikagawa, Y., Kodama, T., Suzuki, E., Kasahara, M., Akiyama, H., Teshima, R., Futo, S., Hino, A., Furui, S., & Kitta, K. (2009). Investigation of residual DNAs in sugar from sugar beet (Beta vulgaris L.). *Journal of the Food Hygienic Society of Japan, 50*(1), 41–46. https://doi.org/10.3358/shokueishi.50.41

Rhee, Y., Shilliday, E. R., Matviychuk, Y., Nguyen, T., Robinson, N., Holland, D. J., Connolly, P. R. J., & Johns, M. L. (2023). Detection of honey adulteration using benchtop 1H NMR spectroscopy. *Analytical Methods, 15*(13), 1690–1699. https://doi.org/10.1039/D2AY01757A

Sajali, N., Wong, S. C., Hanapi, U. K., Abu Bakar @ Jamaluddin, S., Tasrip, N. A., & Mohd Desa, M. N. (2018). The challenges of DNA extraction in different assorted food matrices: A review. *Journal of Food Science, 83*(10), 2409–2414. https://doi.org/10.1111/1750-3841.14338

Shehata, M., Dodd, S., Mosca, S., Matousek, P., Parmar, B., Kevei, Z., & Anastasiadi, M. (2024). Application of Spatial Offset Raman Spectroscopy (SORS) and Machine Learning for Sugar Syrup Adulteration Detection in UK Honey. *Foods, 13*(15). https://doi.org/10.3390/foods13152425.

Soares, S., Amaral, J. S., Oliveira, M. B. P. P., & Mafra, I. (2017). A comprehensive review on the main honey authentication issues: Production and origin. *Comprehensive Reviews in Food Science and Food Safety, 16*(5), 1072–1100. https://doi.org/10.1111/1541-4337.12278

Soares, S., Grazina, L., Costa, J., Amaral, J. S., Oliveira, M. B. P. P., & Mafra, I. (2018). Botanical authentication of lavender (Lavandula spp.) honey by a novel DNA-barcoding approach coupled to high resolution melting analysis. *Food Control, 86*, 367–373. https://doi.org/10.1016/j.foodcont.2017.11.046

Sobrino-Gregorio, L., Vilanova, S., Prohens, J., & Escriche, I. (2019). Detection of honey adulteration by conventional and real-time PCR. *Food Control, 95*, 57–62. https://doi.org/10.1016/J.FOODCONT.2018.07.037

Spiteri, M., Jamin, E., Thomas, F., Rebours, A., Lees, M., Rogers, K. M., & Rutledge, D. N. (2015). Fast and global authenticity screening of honey using 1H-NMR profiling. *Food Chemistry, 189*, 60–66. https://doi.org/10.1016/j.foodchem.2014.11.099

Stefas, D., Gyftokostas, N., Kourelias, P., Nanou, E., Tananaki, C., Kanelis, D., Liolios, V., Kokkinos, V., Bouras, C., & Couris, S. (2022). Honey discrimination based on the bee feeding by laser induced breakdown spectroscopy. *Food Control, 134*, Article 108770. https://doi.org/10.1016/j.foodcont.2021.108770

Taberlet, P., Coissac, E., Pompanon, F., Gielly, L., Miquel, C., Valentini, A., Vermat, T., Corthier, G., Brochmann, C., & Willerslev, E. (2007). Power and limitations of the chloroplast trn L (UAA) intron for plant DNA barcoding. *Nucleic Acids Research, 35*(3), Article e14. https://doi.org/10.1093/nar/gkl938

Tosun, M. (2013). Detection of adulteration in honey samples added various sugar syrups with 13C/12C isotope ratio analysis method. *Food Chemistry, 138*(2), 1629–1632. https://doi.org/10.1016/j.foodchem.2012.11.068

Trifković, J., Andrić, F., Ristivojević, P., Guzelmeric, E., & Yesilada, E. (2017). Analytical methods in tracing honey authenticity. *Journal of AOAC International, 100*(4), 827–839. https://doi.org/10.5740/jaoacint.17-0142

Truong, A.-T., Kim, S., & Yoon, B. (2022). Determination of honey adulterated with corn syrup by quantitative amplification of maize residual DNA using ultra-rapid real-time PCR. *Journal of the Science of Food and Agriculture, 102*(2), 774–781. https://doi.org/10.1002/jsfa.11411

Valentini, A., Miquel, C., & Taberlet, P. (2010). DNA barcoding for honey biodiversity. *Diversity, 2*(4). https://doi.org/10.3390/d2040610. Article 4.

Walker, M. J., Cowen, S., Gray, K., Hancock, P., & Burns, D. T. (2022). Honey authenticity: The opacity of analytical reports—part 2, forensic evaluative reporting as a potential solution. *Npj Science of Food, 6*(1). https://doi.org/10.1038/s41538-022-00127-5. Article 1.

Wang, W., Yang, B., Wang, J., Feng, X., Feng, C., Zhao, T., Shen, L., Wang, Q., Wu, Z., Zhang, S., & Ma, Z. (2020). Molecular and toxicity analyses of white granulated sugar and other processing products derived from transgenic sugarcane. *Frontiers in Plant Science, 11*. https://www.frontiersin.org/articles/10.3389/fpls.2020.596918.

White, J. W. (1992). Internal standard stable carbon isotope ratio method for determination of C-4 plant sugars in honey: Collaborative study, and evaluation of improved protein preparation procedure. *Journal of AOAC International, 75*(3), 543–548. https://doi.org/10.1093/jaoac/75.3.543

Wirta, H., Abrego, N., Miller, K., Roslin, T., & Vesterinen, E. (2021). DNA traces the origin of honey by identifying plants, bacteria and fungi. *Scientific Reports, 11*(1), 4798. https://doi.org/10.1038/s41598-021-84174-0

Wu, Y., Yang, Y., Liu, M., Wang, B., Li, M., & Chen, Y. (2017). Molecular tracing of the origin of six different plant species in bee honey using real-time PCR. *Journal of AOAC International, 100*(3), 744–752. https://doi.org/10.5740/JAOACINT.16-0265

Xiujie, Z., Wujun, J., Wentao, X., Xiaying, L., Ying, S., Sha, L., & Hongsheng, O. (2019). Comparison of five endogenous reference genes for specific PCR detection and quantification of rice. *Rice Science, 26*(4), 248–256. https://doi.org/10.1016/j.rsci.2019.04.005

Xue, X., Wang, Q., Li, Y., Wu, L., Chen, L., Zhao, J., & Liu, F. (2013). 2-Acetylfuran-3-Glucopyranoside as a novel marker for the detection of honey adulterated with rice syrup. *Journal of Agricultural and Food Chemistry, 61*(31), 7488–7493. https://doi.org/10.1021/jf401912u

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics, 13*, 134. https://doi.org/10.1186/1471-2105-13-134

Ždiniaková, T., Loerchner, C., De, R. O., Dimitrova, T., Kaklamanos, G., Breidbach, A., Respaldiza, H. M. A., Vaz, S. I. M., Paiano, V., Ulberth, F., & Maquet, A. (2023). EU Coordinated action to deter certain fraudulent practices in the honey sector. *JRC Publications Repository*. https://doi.org/10.2760/184511

Aries, E., Burton, J., Carrasco, L., De Rudder, O., & Maquet, A. (2016). Scientific support to the implementation of a Coordinated Control Plan with a view to establishing the prevalence of fraudulent practices in the marketing of honey. *JRC Technical Report*, 1–33, 6932951. JRC104749.

JRC. (2006). Event-specific method for the quantitation of rice line LLRICE62 using real-time PCR. Community Reference Laboratory for GM Food and Feed. CRLVL05/04VR, 1-12. Available online: https://www.infogm.org/IMG/pdf/ccr_1507_59122_20060613.pdf. (Accessed 11 December 2023).

Honey (England) Regulations. (2015). The honey (England) regulations 2015. King's Printer of Acts of Parliament. No. 1348. Available online: https://www.legislation.gov.uk/uksi/2015/1348/made. (Accessed 14 May 2024).



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